Encapsulation, Color Stability, and Distribution of Anthocyanins from Purple Corn (*Zea mays* L.), Blueberry (*Vaccinium* sp.), and Red Radish (*Raphanus sativus*) in a Cold-Setting Pectin-Alginate Gel.

THESIS

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Abstract

Anthocyanins are a broad class of water soluble pigments found in a wide array of plants. They are responsible for a variety of the attractive colors found in fruits including red, purple, orange, and blue. There use as a natural alternative to synthetic colorants has been investigated extensively in the past several years as consumers have been asking for greater choice in the marketplace. Their limited stability in food applications several limits their widespread adoption. Meanwhile, other research has been focused on the potential health promoting benefits of eating a diet high in anthocyanins.

The objective of this work was to encapsulate an anthocyanin rich extract in a novel system that uses pectin and alginate as the encapsulating material in hopes to increase stability. It was then necessary to investigate not only the color stability of the particles, but also the anthocyanin stability and profile as well.

A variety of anthocyanin sources was chosen to represent a cross section of the structural differences that exist: blueberry (5 of the 6 common anthocyanidins all with a single, varying sugar moiety), purple corn (3 monoglycosylated anthocyanins and their malonated counterparts), and red radish (pelargonidin derivatives with 3 glycosylations and aromatic acids). All three anthocyanins sources were successfully encapsulated using the technique described later. The stability of the particles was monitored by increasing color of the solutions the particles were stored in, the color of the particles themselves, and the total monomeric anthocyanin content of both.

The purple corn particles performed the best in regards to color leaching into the solution. The solution color indices for the experimental and control were more different than for the other anthocyanin sources tested. This performance carried over when measuring the color of the gels directly. The purple corn loaded gels were statistically darker and had greater color intensity than the empty control gels. The red radish loaded gels performed the worst for every measurement that was taken.

The interesting difference was noted in the amount of anthocyanin recovered from each gel. The amount of anthocyanin leached into the solutions was not different among the sources. Another interesting note was that the anthocyanin profile of the various extracts did not change significantly during the storage study, meaning that preferential leaching or retention in the gel was not noted.

The pectin-alginate system was able to encapsulate various anthocyanin rich extracts, with the purple corn performing the best of those tested, with the blueberry performing similarly. Since these anthocyanins are relatively small compared to others, it is theorized that molecule size is not the main factor contributing to pigment retention. It is possible that other anthocyanin sources will work just as well.

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Fields of Study

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1 Literature Review

1.1 What is Color?

Color is the first characteristic that a person notices when judging a food. Although other characteristics of a food (e.g. flavor, texture, nutritiousness) are important in the overall development, perhaps none can influence how a person perceives a food as much as color (Wrolstad and Smith 2010). Color, as it is perceived by humans, is the reflection or transmission of various wavelengths of light off of or through an object that are detected by the eye. Fig. 1 provides a basic illustration of this concept. The combination of different wavelengths at varying intensities is what accounts for the almost 10 million different colors that humans can distinguish (Judd and Wyszecki 1975).

Since the human eye doesn't detect specific wavelengths but instead looks at the entire spectrum of visible light, it is important to have color systems that do the same thing. Several color spaces have been created that allow for reproducible measurements that can be mathematically compared. The CIE L*a*b* color space is one of the more common color spaces used. In this system, L* is the lightness value, a* is the redness, and b* is

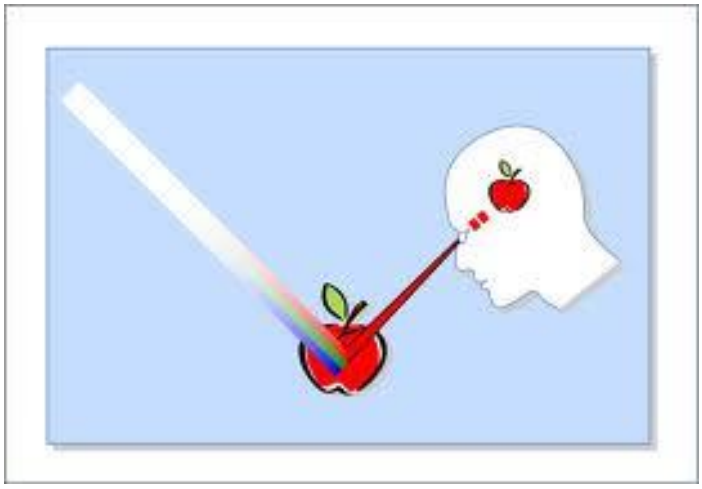

Figure 1: A light source, object, and observer source
http://farm5.staticflickr.com/4117/4818335835_9f226cf4d8_z.jpg

the yellowness of the color. Another similar system is CIE L*C*h that uses the color intensity (chroma) and the hue angle. Fig 2 shows how those 2 color spaces are related.

1.2 Food Color: History & Importance

Many foods have what is considered a characteristic color that is not natural to that food, but is instead added to it. Cheese and butter are naturally a yellowish, off-white color but often have an orange-yellow colorant added to them to provide a uniform color all year round. Although many would consider this to be a modern adaptation meant to deceive consumers, there are records that show that colorants have been added to food for more than 3000 years (Burrows 2009; Sharma, McKone, and Markow 2011). Throughout the years, natural and synthetic dyes have been added to food for a variety of reasons including but not limited to: (1) compensating for seasonal variations in a product, (2) improve the color of the product as it sits on a shelf, (3) give the characteristic color that

corresponds with a flavor, and (4) make up for color that may be lost during processing or storage.

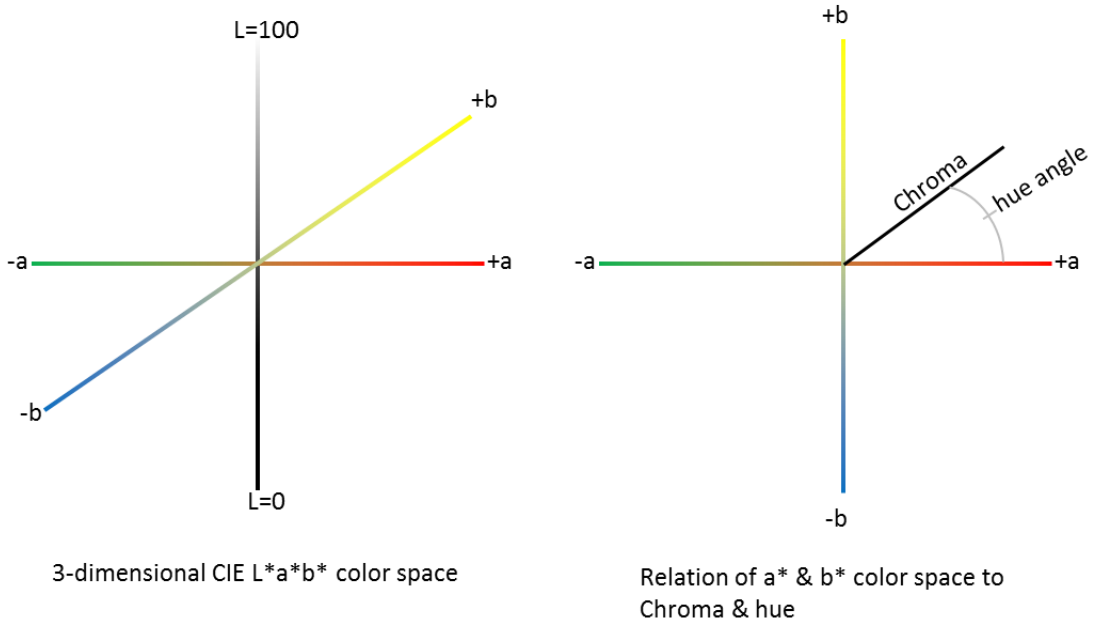

Figure 2: CIE L*a*b* color space and how it relates to chroma and hue.

The original color of a food can tell a great deal about what it is, its stage of maturation, authenticity, safety and how concentrated it is. A great example of this is with the banana. As a banana progresses from being unripened to ripened and then to overripened the color on the outside changes from green to yellow to brown. Most small children can identify this in a banana without having to touch it, demonstrating just how powerful the color of a food can be. The orange, blue, or green colors that many molds produce are a clear sign that the food in question has spoiled. One doesn't need to eat, smell or even touch the food to know that they should not consume it.

Whenever a color is added to a food, whether it is natural or synthetic, it falls under the supervision of the Food and Drug Administration (FDA) in the United States. The regulations that cover these compounds are found in Title 21 of the Code of Federal Regulations.. They were originally classified as food additives until the Color Additive Amendments of 1960 were passed.

The FDA groups food colorants into 2 categories: those that require certification and those that are exempt from certification. Those that require certification are properly referred to as FD&C colorants but colloquially are referred to as artificial or synthetic. These colorants are given a classification such as FD&C Blue no. 1 but may also be referred to by its common name of Brilliant Blue FCF. There are currently 9 colors that require certification and they are listed in Table 1. Every batch of an FD&C colorant must be tested and certified by the FDA before it can be added to a food.

Title 21 subsection	Legal Classification	Common Name
74.101	FD&C Blue No. 1	Brilliant Blue FCF
74.102	FD&C Blue No. 2	Indigotine
74.203	FD&C Green No. 3	Fast Green FCF
74.250	Orange B.	n/a
74.302	Citrus Red No. 2	n/a
74.303	FD&C Red No. 3	Allura Red AC
74.340	FD&C Red No. 40	Erythrosine
74.705	FD&C Yellow No. 5	Tartrazine
74.705	FD&C Yellow No. 6	Sunset Yellow FCF

Table 1: List of current FD&C colorants

The remaining food colorants are those that do not require FDA certification before being sold. The majority of these are juices, purees, or extracts from fruit or vegetable sources. The regulations concerning these colorants can be found in Title 21 Part 73 of the Code of Federal Regulations (CFR).

Colorants can also be classified using several different systems; once of which is to classify them by their source. This typically results in 4 classes(Mortensen 2006; Mateus and Freitas 2008):

a) Natural Colors: Those colors that are derived from edible plant and animal sources. e.g. Betalains, caramel colors, turmeric

b) Nature Identical Colors: Those compounds that are chemically identical to natural colors but are created via chemical synthesis. e.g. β-carotene

c) Synthetic Colors: Those compounds that are not found naturally in nature and are the result of chemical synthesis. e.g. Allura red, tartrazine

d) Inorganics: Those compounds that are mined or synthesize primarily, compounds of metals. e.g. Titanium dioxide

1.3 Anthocyanins

1.3.1 Background

Anthocyanins are a very diverse class of water soluble pigments. The color can range from bright oranges and reds to deep purples and blues. Many different factors contribute to the variety of colors and those will be discussed later. The diversity of anthocyanins is not only the different colors they can produce, but also the wide array of

plant materials that they are found in including leaves, fruits, flower, and roots. The word anthocyanin is itself a combination of the Greek words for blue and flower (Delgado-Vargas and Paredes-Lopez 2003).

In addition to the vibrant colors that they provide in foods, research performed over the past few decades has shown that they may have health promoting properties as well. It is believed that in addition to providing color that attracts pollinating animals, the pigments are able to protect the plant from damaging reactive species by functioning as an antioxidant. Research has shown that red wine, a rich source of anthocyanins, is able to protect human blood cells from damaging reactive oxygen species (Tedesco et al. 2001). Anti-inflammatory properties have been demonstrated by several recent studies (Wang et al. 1999; Seeram et al. 2001; Rossi et al. 2003). Preventing inflammation could play a very key role in preventing the initial stages of cancer. *In vitro* cell studies have also been undertaken to judge the effectiveness of anthocyanin rich extracts on cell proliferation. Very promising results have been achieved with gastric cells (Kamei et al. 1998), colon cells (Yi et al. 2005), and oral cells (Rodrigo et al. 2006).

1.3.2 Structure

Anthocyanins are a very large class of polyphenolic compounds that belong to the group known as flavonoids. Like all flavonoids, anthocyanins have a basic structure of a 15 carbon skeleton in a C-6 C-3 C-6 configuration. The first six and last six carbon atoms each form a phenolic ring that are referred to as the A and B rings respectively. This

most basic form is referred to as an anthocyanidin. There are several points of substitution along this that determines the exact name for the anthocyanidin. At least 18 different anthocyanidins have been discovered as of 2012 (Skaar et al. 2012). Of those 18, there are six that occur most often in food materials: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Their structure and substitutions are displayed in Figure 3 and Table 2.

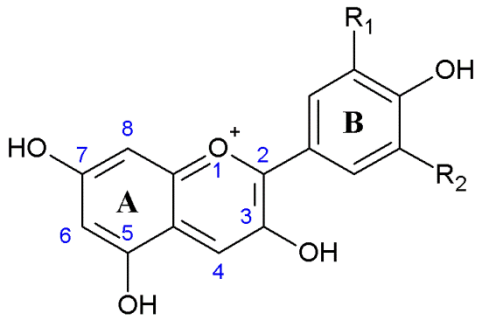

Figure 3: Basic structure of the six common anthocyanidins found in food materials

Name	Substitution		λmax (nm)	Molar Mass (g)
	R_1	R_2		
Pelargonidin	H	H	494	271
Cyanidin	OH	H	506	287
Peonidin	OCH_3	H	506	301
Delphinidin	OH	OH	508	303
Petunidin	OCH_3	OH	508	317
Malvidin	OCH_3	OCH_3	510	331

Table 2: 6 Major anthocyanidins, their substitutions, maximum absorbance, and molar mass.

From these basic aglycones, more than 650 anthocyanins have been discovered naturally occurring in various animal, plants, and microorganisms (Skaar et al. 2012). This is due

to numerous glycosylations and acylations combinations that occur (Wrolstad 2004). More often than not, one or more sugar moieties are attached via a glycosidic bond at the 3-, 5-, and 7-position. The sugar moieties that occur with the greatest frequency are the monosaccharides glucose (glu) and rhamnose (rha). Other sugars that are still common but occur less regularly are the monosaccharides galactose (gal), arabinose (ara), and xylose (xyl) and the disaccharides rutinose (rut) and sambubiose (sam) (He and Giusti 2010). Some glycosylated anthocyanins may also be acylated with aromatic and/or aliphatic acids. These organic acids are customarily attached through an ester linkage on the sugar moiety. Figure 4 shows 12 of the most common organic acids found in anthocyanins.

The various differences among anthocyanins i.e. glycosylation, acylation, B-ring substitutions all play a role in perceived color and stability of the pigment (Giusti, Rodríguez-Saona, and Wrolstad 1999). Light, pH, heat, and redox conditions are all harmful to the stability of the anthocyanins. If these conditions are optimal for the anthocyanins, the aglycone form will be less stable than a glycosylated form which is in turn less stable than the acylated form. Several complimentary explanations have been developed to suggest why this is the case. A brief examination of the anthocyanin structure shows at least 7 hydroxyl groups on monoglycosylated anthocyanin. For anthocyanins with di- or triglycosylations and acylating groups, the number of possible hydrogen bonding sites increases. It has been suggested (Dangles, Saito, and Brouillard 1993a; Dangles, Saito, and Brouillard 1993b; Brouillard and Dangles 1994; Giusti,

Rodríguez-Saona, and Wrolstad 1999) and demonstrated using nuclear magnetic resonance (NMR) (Goto 1987; Giusti, Ghanadan, and Wrolstad 1998; Borkowski et al. 2005) that complex anthocyanins are able to form intramolecular hydrogen bonds between the anthocyanidin, the sugar moiety and the acylating group. These interactions

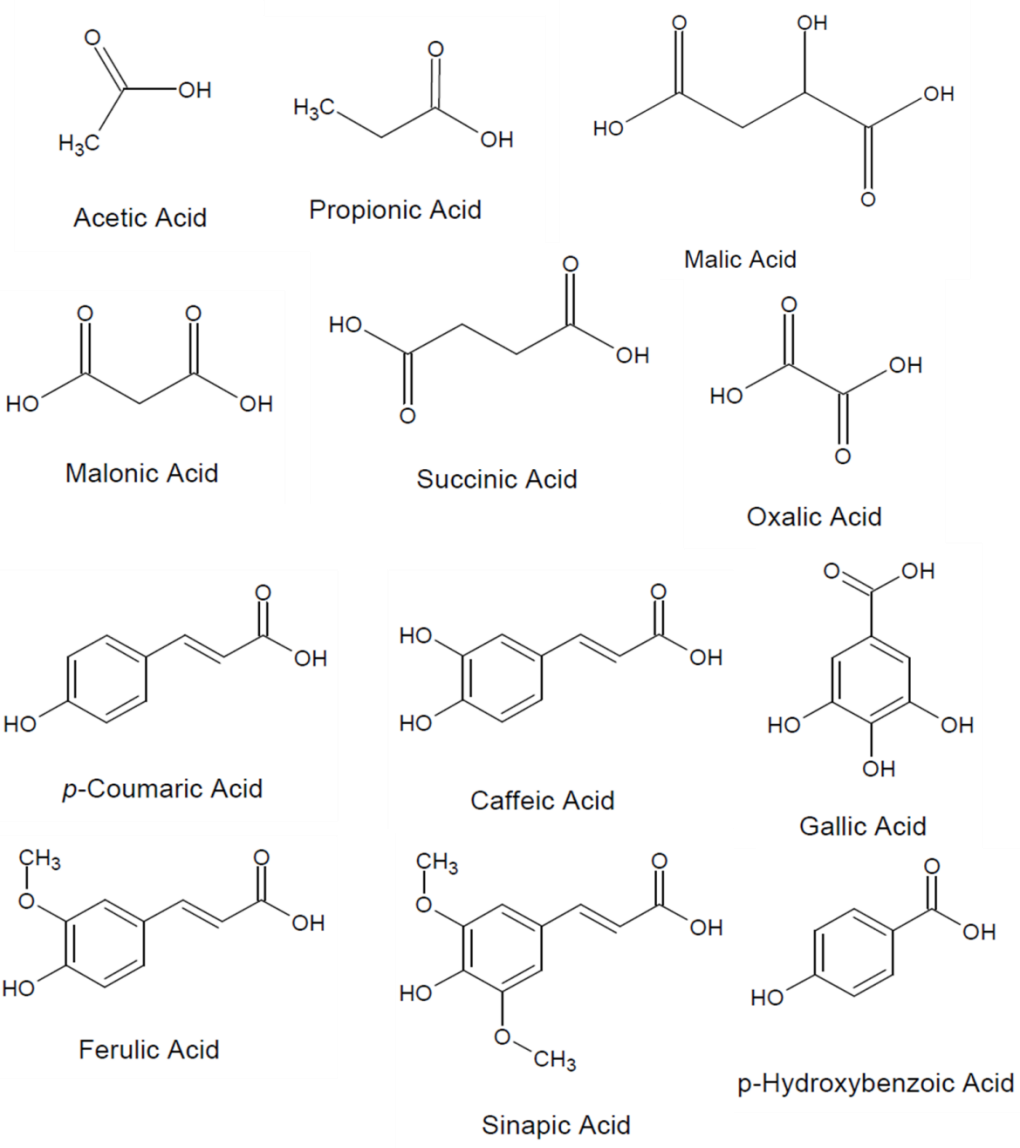

Figure 4: Common acylating groups found on anthocyanins

have been referred to as folding. The anthocyanins are also capable of forming intermolecular hydrogen bonds with other anthocyanins or similar phenolic compounds. These intermolecular interactions are referred to as either copigmentation or stacking. The theory behind the increased stability as that through this network of phenolic groups and hydrogen bonds, the severity of an electron loss or radical formation can be spread out over the entire molecule or molecules. It is also possible that the spatial reorientation that is undergone as a result of the bonding creates a new configuration which is inherently more stable. The proposed configurations of folding and stacking are shown in Figure 5.

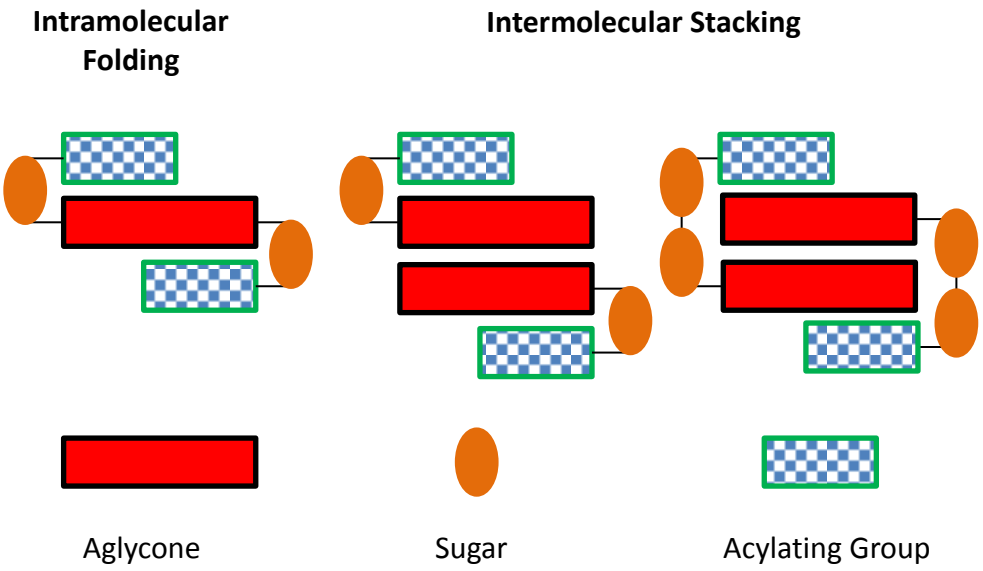

Figure 5: Intra and intermolecular associations of acylated anthocyanins. Adapted from (Yoshida, Kondo, and Goto 1991; Giusti and Wrolstad 2003)

1.3.3 Spectral & Color Variations

In acidic conditions, all anthocyanins are a shade of orange-red when at the same concentrations. Pure pelargonidin has a maximum absorbance around 495nm depending on the solvent conditions. As shown back in Table 2, the addition of hydroxyl and methoxyl groups on the B-ring result in a bathochromic shift in the maximum absorbance. A bathochromic shift is when the wavelength of maximum absorbance increases. The end result is that the appearance of the anthocyanin shifts away from orange towards purple-blue. This bathochromic shift is even more apparent when the anthocyanin is glycosylated and acylated (Giusti and Wrolstad 1996a; Giusti, Rodríguez-Saona, and Wrolstad 1999; Stintzing et al. 2002; Giusti and Wrolstad 2003). Figure 6 demonstrates the shift that occurs when pg-3-soph-5-glu, the common anthocyanin found in red radishes, has either 1 or 2 acylating groups present.

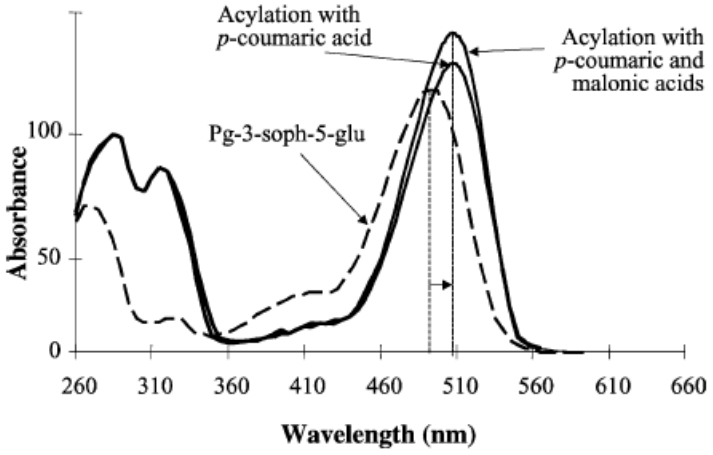

Figure 6: Bathochromic and hyperchromic shift of pelargonidin-3-sophoroside-5-glucoside with different acylating groups. Source: (Giusti and Wrolstad 2003)

In addition to the bathochromic shift, a hyperchromic shift is also noted in Figure 6. A hyperchromic shift is when the absorbance value increases for a constant molar quantity. The position and number of glycosylations will also play a role in the spectra of a given molecule. A monoglycosylated anthocyanin will have a "shoulder" in the 440nm range. It was first reported by Harborne that the number glycosidic substitutions could be determined by analyzing the spectra (Harborne 1967). This was further confirmed by Giusti by comparing the spectra of pelargonidin-3-glucoside and pelargonidin-3-sophoroside-5-glucoside, shown in Figure 7 (Giusti and Wrolstad 1996a).

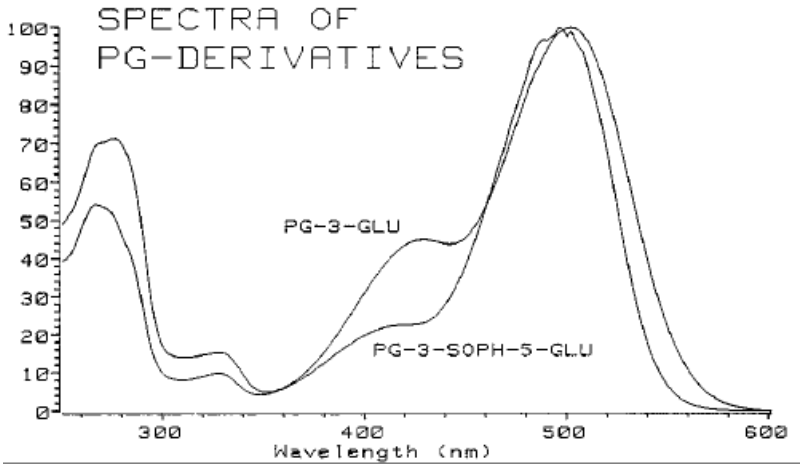

Figure 7: Comparative spectra of alkaline hydrolyzed anthocyanins. Source: (Giusti and Wrolstad 1996a)

1.3.4 pH related changes

While glycosylation and acylation play a role in the color of an anthocyanin, perhaps the most dramatic color changes are those that are pH dependent. (Dangles, Saito, and

Brouillard 1993a) At pH 1, anthocyanins in solution are a deep, vibrant orange-red color. As the pH approaches 4.5, the solution becomes almost colorless. The color then shifts toward blue, green, or purple as the pH goes above 7. This is due to the structural changes that occur to the anthocyanin molecule as a result of the pH. The anthocyanin structure shown in Figure 3 is referred to as the flavylium cation. This is the predominant form at pH 1. At pH 4.5, the anthocyanin becomes one of two uncharged forms: carbinol, pseudo-base, or chalcone. Both of these forms cause a change to the molecules chromophore and therefore these molecules are essentially colorless. The final form that anthocyanins undergo is the quinonoidal base. This is the dominant form above pH 7 and is normally bluish, but this color can appear as green or purple depending on the specific anthocyanin and environmental conditions. Although in Figure 8, only one form of quinonoidal is shown, at least 3 mesomeric forms are known to exist.

1.4 Stability

As previously discussed, the structural differences among the various anthocyanins play an important role in the overall stability of the pigment. The more simple structures tend to degrade faster while the larger pigments tend to be more stable. Even within fruits that have similarly structured anthocyanins, stability can vary. Various juices from grape, bilberry, plum, strawberry, and a few others were stored at 20°C for more than 4 months to determine their degradation kinetics. (Hernández-Herrero and Frutos 2011) The anthocyanins from strawberry had a half-life of less than 3 weeks, while the grapes were almost 10x that with a half-life of 23.6 wks.

Elevated temperatures play a major role in the stability as well. Blackberry juice (Wang and Xu 2007) and black carrot extract (Kırca, Özkan, and Cemeroğlu 2007) were both subjected to temperatures varying temperatures to determine how much of a factor that played. In both cases, a temperature increase of 10°C resulted in the half-life of the anthocyanins being cut in half. Light also plays a major role in the color stability of anthocyanins. Purple corn and sweet potato were subjected to direct light treatments for 10 days. (Cevallos-Casals and Cisneros-Zevallos 2004) The sweet potato proved to be more stable than the purple corn, but both were much less stable than their controls.

1.5 Sources of Anthocyanins

The overwhelming majority of blue-purple-red plants owe their vibrant colors to the presence of anthocyanins. With over 650 having been identified, the exact profile can vary greatly from species to species. Fruits tend to have anthocyanins that have simple, mono- or diglycosylations while vegetables tend to have multiple glycosylations and acylations. The amount can also vary greatly. Strawberries have 1 predominant anthocyanin while grapes have many. These differences can be used to identify unknown extracts and can serve as a 'fingerprint' of sorts.

Blueberries (*Vaccinium* sp) have a diverse anthocyanin profile that can vary from cultivar to cultivar. Up to 24 different anthocyanins have been reported (Barnes et al. 2009), but

many of those occur in levels that are so low that they are below the detection limit for many extraction and identification methods. Most analyses of blueberry report between

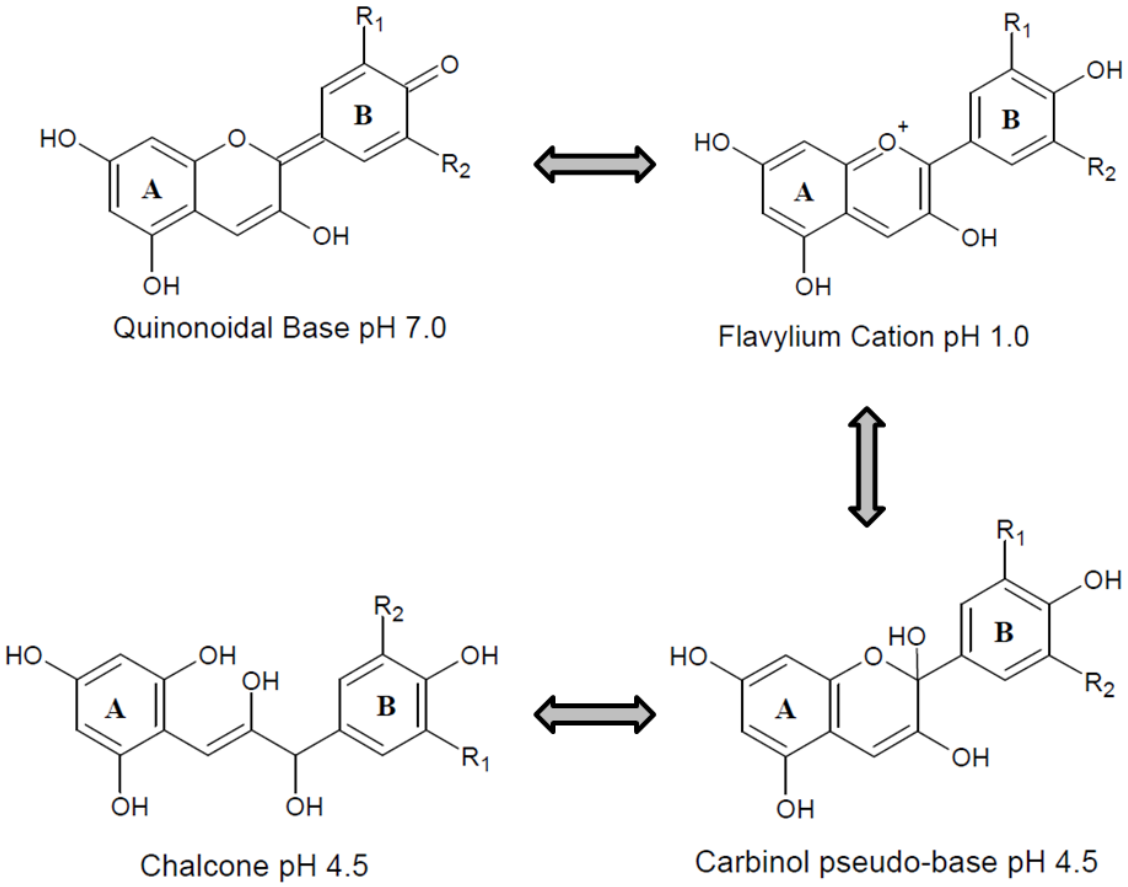

Figure 8: Structural changes of the anthocyanin molecule as a result of changes in the pH. Adapted from:(Dangles, Saito, and Brouillard 1993a)

10 and 15 distinct anthocyanins (Skrede, Wrolstad, and Durst 2000; Lee, Durst, and Wrolstad 2002; Seeram et al. 2006; Bae et al. 2009; Wang, He, and Li 2010; Del Bo et al. 2012). When 15 are reported, they are cyanidin, delphinidin, peonidin, petunidin, and malvidin monoglycosylated with glucose, galactose, and arabinose. (Figure 9)

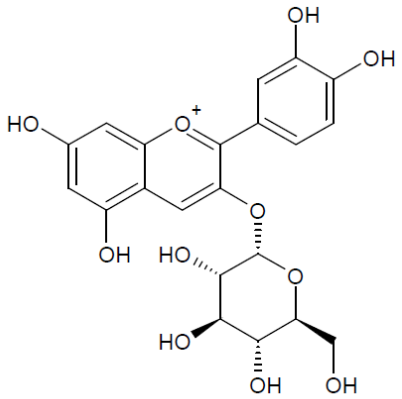

Figure 9. Structure of cyanidin-3-glucoside

Purple corn (*Zea mays* L.) is a variety of corn native to South American that has high levels of anthocyanins. The cob has been used as a colorant in South American for centuries(Jing et al. 2007). Its profile consists of cyanidin, pelargonidin, and peonidin glucosylated in the 3 position and their 6" malonated counterparts (Figure 10) (De Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo 2002; Cevallos-Casals and Cisneros-Zevallos 2004; Jing and Giusti 2005; Jing et al. 2007; Jing and Giusti 2007).

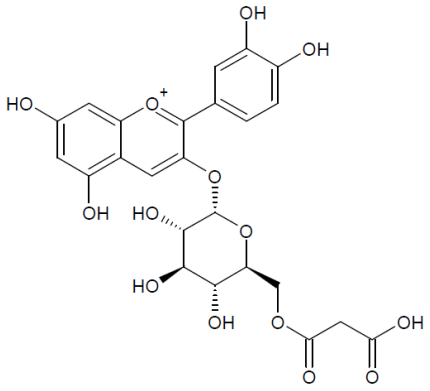

Figure 10. Structure of cyanidin-3-(6-malonyl)glucoside

Red radish (*Raphanus sativus* L.) has an outer layer that is rich in anthocyanins. The anthocyanins are pelargonidin-3-sophoroside-5-glucoside derivatives that are acylated

with cinnamic acids and malonic acid (Giusti and Wrolstad 1996a). (Figure 11 The exact acylations and profile vary by cultivar. Some of the more common cinnamic acids are ferulic, coumaric, and caffeic acid. There has been increased interested in these pigments lately due to their potential use as a replacement for FD&C Red #40 (Giusti and Wrolstad 1996b; Giusti et al. 1998; Rodríguez-Saona, Giusti, and Wrolstad 1999). The red radish pigments naturally provide a color that is very similar to FD&C Red #40 and due to the triglycosylation and acylating groups, they have greater stability than most other natural red colorants.

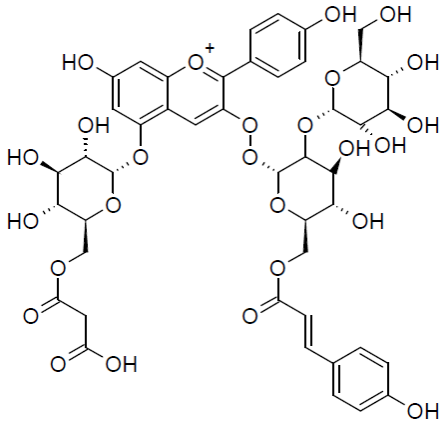

Figure 11. Structure of pelargonidin-3-coumaroyl-sophoroside-5-malonyl-glucoside

1.6 Encapsulation

Encapsulation is a technique wherein an active compound is mixed with a carrier agent (or wall material). The active compound (sometimes called core material) can be a flavor, drug, or pigment to name a few. The possible carrier agent is dependent upon the active compound, technique used, and reason for encapsulating. Sugars, proteins, polysaccharides, and gums are all common encapsulating material. There are many

reasons why a company may wish to encapsulate a product, including but not limited to protecting from environment induced decay, mask undesirable taste, control release of the core material, and increase solubility (Shahidi and Han 1993).

There are several techniques that are currently available for encapsulating. Perhaps the most common is spray drying. Having been around since the 1950's, it is typically used to make stable, dry food additives (Desai and Jin Park 2005). Spray drying involves making a homogenous mixture of the core material and wall material, which is then atomized inside a spray dryer (Gibbs et al. 1999). The dried particles tend to be spherical with an average diameter of 10-100 μm. While carbohydrates (maltodextrins, starches, pectin) are the most commonly used materials, they are not the only option available today. Gums and proteins have gained interested due to the unique functional properties and interactions with the core material that they can have (Gharsallaoui et al. 2007).

1.7 Anthocyanin Encapsulation

The inherent instability of natural pigments has limited their adoption as a wide spread food colorant. A considerable amount of research in the past 10 years has been done on finding ways to increase the stability of natural colorants. Since anthocyanins are water soluble, they tend to be ideal for spray drying techniques.

The ethanolic extract containing black carrot anthocyanins was encapsulated using maltodextrin (MD) as the wall material. These spray dried particles ranged in size from

3-20 µm (Ersus and Yurdagel 2007). Anthocyanins purified from corozo (*Bactris guineensis*) were also encapsulated using maltodextrin. These spray dried particles were larger, but were still less than 50 µm (Osorio et al. 2010). The size difference is most likely due to the different feed rates and mixture temperatures that were used by the different researchers. Pomegranate juice and ethanol extracts were encapsulated using either maltodextrin or soy protein isolate (SPI). The encapsulation efficiency for anthocyanins was greater for the MD spray dried particles, but stability was greater for the SPI particles (Robert et al. 2010). A 1:1 ratio of modified starch and maltodextrin was also used to encapsulate pomegranate juice (Nogueira et al. 2011). The spray drying yield and efficiency were found to not be dependent upon the processing parameters that were investigated. A crude extract from a member of the mangosteen family, *Garcinia indica*, was encapsulated using maltodextrins of varying dextrose equivalents(DE), along with other additives, to determine an optimal core material (Nayak and Rastogi 2010). A combination of MD 21 DE, gum acacia, and tricalcium phosphate was able to produce spray dried particles that were less than 50 µm and high in anthocyanin content. Cabernet Sauvignon grapes (*Vitis vinifera* L.) were extracted in an acidified ethanol and water mixture. Maltodextrin was used as the wall material and was combined with either cyclodextrin or gum Arabic to try and optimize a spray dried particle (Burin et al. 2011). The combination of maltodextrin and gum Arabic produced colored particles with the longest half-life and spherical particles that were less than 50 µm in size. An ethanolic anthocyanin extract from blueberries (*Vaccinium ashei* L. Rabbiteye) were successfully encapsulated using mesquite gum as the wall material (Jiménez-Aguilar et al. 2011). Not

surprisingly, hotter drying temperatures had a negative effect on the color stability of the anthocyanins.

While spray drying is the most used technique for encapsulating anthocyanins, there have been other techniques. Anthocyanins from hibiscus (*Hibiscus sabdariffa*) have been encapsulated using multiple techniques. They have been mixed with pullulan, a starch of fungal origin, and freeze dried (Gradinaru et al. 2003). The stability of the anthocyanins was found to be directly related to the water activity at which it was stored. Water activities above 0.53 were found to severely decrease stability. Similar results were found when maltodextrin and gum Arabic were used (Selim et al. 2008). Bilberries (*Vaccinium myrtillus*), a European plant similar to the North American blueberry, have been researched extensively. Thermo set gels were created by heating a solution of bilberry anthocyanins and acidic whey protein isolate. Higher protein levels and lower anthocyanin concentrations resulted in the lowest possible loss of anthocyanin when placed in a simulated gastric fluid (Betz, Tolkach, and Kulozik 2009; Betz and Kulozik 2011a). These findings were using to generate microencapsulated bilberry through an emulsion/heat gelation method (Betz and Kulozik 2011b). These researchers were able to create microparticles smaller than 70 µm by adjusting the emulsifier used and increasing the RPM on the mixer. This technique was then compared against a traditional spray drying technique and a technique using pectin capsules, cross linked by a calcium chloride solution (Oidtmann et al. 2012). When placed in a simulated gastric fluid, all

three behaved similarly in terms of degradation and release from the core material. All three had superior stability when compared to unencapsulated bilberry anthocyanins.

1.8 Pectin and Alginate

1.8.1 Pectin & Alginate Structure

Pectin is perhaps one of the most well-known polysaccharides and food gums used today. It is naturally found in a wide variety of foods, especially grapes, apple, and citrus peels (Brejnholt 2011). Commercially available pectin comes primarily in two forms: high methoxy and low methoxy. Both of these types have the same backbone of galacturonic acid. The structural difference between the two is in how often the carboxylic acid group on the C6 of galacturonic acid has a methyl group. If more than 50% of the acid groups are methylated, it is high methoxy (Figure 12) and if less than 50%, low methoxy pectin. This structural difference is responsible for the operational differences that must be accounted for when using the different types of pectin as a gelling agent. High methoxy pectin forms a very strong gel when there are high soluble solids (>55%) and a pH of below 3.6. These requirements are almost the exact characteristics for jelly and jam. Gelling characteristics are quite different for the low methoxy pectin. The free carboxylic groups that are prevalent will be deprotonated above pH 3.6. Those negative groups will form bonds with cations. Divalent calcium is often used to cross link neighboring pectin strands. This allows for a stable gel without the addition of sugar.

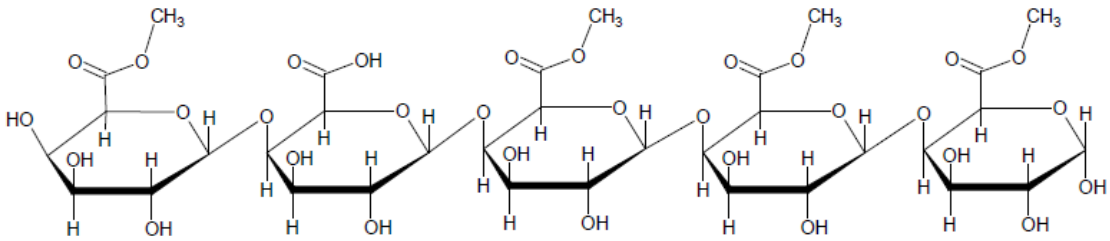

Figure 12. Representative structure of high methoxy pectin

Alginate (alginic acid) is a polysaccharide that is extracted from some types of seaweed. (Figure 13) It is comprised of blocks of mannuronic and guluronic acids and can have a molecular weights up to 600 kDa (Helgerud et al. 2011). It has free carboxylic groups very similar to those of low methoxy pectin.

Both common types of pectin and alginate are soluble in water. They can thicken and increase the viscosity when in solution, but require the addition of another substrate to form a gel.

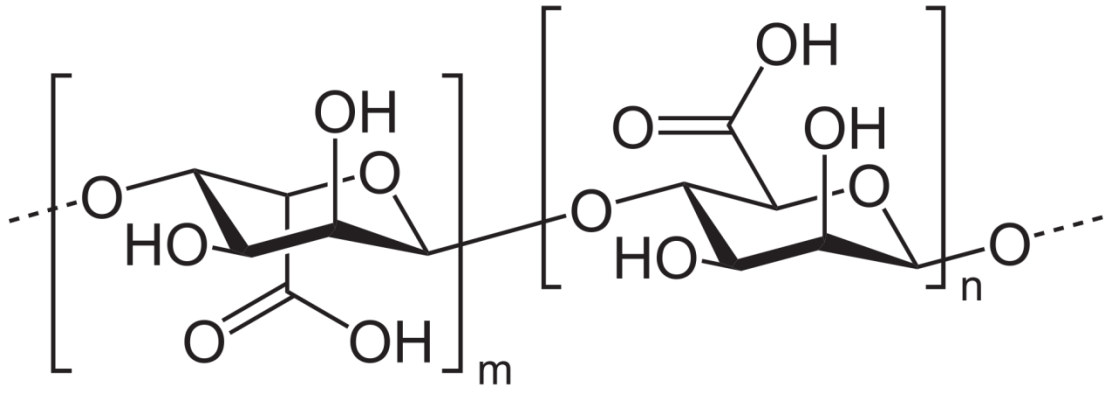

Figure 13. Structure of alginic acid. m-mannuronic acid n-guluronic acid

1.8.2 Synergistic Alginate-Pectin Gelation

Although neither alginate nor pectin alone forms gel without calcium ions or presence of sugar respectively, their mixtures can form gels at low pH (Toft, 1982). The spontaneous gelling that occurs when a mixture of high methoxy pectin and alginate are combined in a mixture with a pH below 4.0 is an interesting interaction. The gelling is independent of sugar level and more closely linked to the blocks of mannuronic and guluronic acid (Toft 1982). These interactions were further investigated and it was found that high levels of guluronic acid residues along with pectin with high methoxy regions created optimal conditions for gelation (Thom et al. 1982). When these regions were in close proximity at pH below 4, whether that pH was the result of quick acidification or slower dialysis, the chains will associate with each other and form a firm gel. The majority of research done until this point had involved heating the solution, acidifying it, and then letting it cool. It was upon cooling that the gel would set up. It wasn't until 1984, that a method was developed that would allow for gelation without heating. Glucono-δ-lactone (GDL) was added to decrease the pH of the solutions from above 6 to below 3 (Morris and Chilvers 1984). When added to either a pectin only or alginate only solution, no gelation would occur. A solution of 1.5% alginate and 1.5% pectin will undergo gelation in less than 30 minutes or when the pH dropped below 3.4. These results were confirmed and expanded upon (Toft, Grasdalen, and Smidsrod 1986). Toft and others showed that the addition of divalent calcium upon gelation resulted in a stronger gel.

1.8.3 Existing Pectin-Alginate Systems

The combination of alginate and pectin has been used as the wall material for encapsulation of vitamin C and anthocyanins (Higuita-Castro et al. 2012) and for folic acid (Madziva, Kailasapathy, and Phillips 2005, 2006). Folic acid encapsulation involved mixing together various ratios of pectin and alginate and then adding folic acid to that mixture. This mixture was dripped through a nozzle into a calcium chloride solution, allowed to harden, and then freeze dried. A mixture of 70:30 alginate to pectin tended to be the best in regards to slower release in a medium, folic acid retention during prolonged storage, encapsulation efficiency and particle size. This system was used to fortify cheese by adding the encapsulated folic acid to the milk, during the cheddaring process, and into the pressed cheese block (Madziva, Kailasapathy, and Phillips 2006). The encapsulated folic acid was shown to be stable to the initial acidification step that occurs during cheese making. By placing the particles in the milk before cheese making as opposed to later on during the process, the particles were able to be evenly distributed throughout the cheese curd. The folic acid capsules were also shown to be stable during 3 months of storage. The micro particles of alginate-pectin containing anthocyanins or vitamin C were prepared by slowly acidifying the mixture using glucono-delta lactone (Higuita-Castro et al. 2012). The gelling formulation was optimized for desired gelling time based on studies of rheological parameters and pH of the mixtures as a function of time.

Objective

The objective of this work was to encapsulate an anthocyanin rich extract in a novel pectin and alginate system to increase stability. No published literature could be found demonstrating the use of this unique system for comparing anthocyanins and their color stability, making its research very valid. The behavior of the particles and how they interact with the anthocyanins were unknown. It was therefore necessary to investigate not only the color stability of the particles, but also the anthocyanin stability and profile as well.

It is hypothesized that the anthocyanins from all of the sources can be encapsulated. The large molecular weight pigments found in the red radish should stay in the gels better due to their large size. The open, porous network of the pectin-alginate system will allow some pigments to leach out, but the larger ones should leach less.

2 Materials & Methods

2.1 Materials

All chemicals and reagents, unless otherwise specified, were from Fisher Scientific. Anthocyanins from three different sources were used. A powdered extract made from purple corn (*Zea mays* L.) was supplied by Peruvian Agroindustries (Lima, Peru). A concentrated red radish (*Raphanus sativus*) extract was supplied by Synergy (Wauconda, IL). A blueberry (*Vaccinium* sp.) juice concentrate was provided by SVZ (Othello, WA). Stock solutions were made for each type by adjusting to 1000mg of anthocyanin/L using 0.01% acidified ethanol with HCl. The sodium alginate used was FMC BioPolymer (Philadelphia, PA) Protanal SF120RB. The pectins used were both provided by Tic gums (Belcamp, MD). They were Pretested® Pectin HM Rapid Set (RSHM Pectin) and Pretested® Pectin HM Slow Set (SSHM Pectin).

2.2 Methods

2.2.1 Gel Preparation & Storage

The gel formulation was modified from Higuita-Castro 2012, in collaboration with the Kaletunc Lab in the Food, Agricultural, and Biological Engineering Department at The

Ohio State University. A 2% (w/w) sodium alginate solution was created by mixing the powder with deionized water using a high shear mixer. A 4% (w/w) solution of both the SSHM and RSHM pectins were created in the same manner. All solutions were allowed to rest overnight in a 4°C refrigerator before use. Alginate (7.0g), SSHM pectin (1.5g), and RSHM pectin (1.5g) solutions were mixed together. 1.0mL of the anthocyanin stock solution was mixed in. A freshly prepared solution of 13% (w/w) glucono-δ-lactone in deionized water was then added. The final composition of the various gels can be found in Table 3. This homogeneous mixture was poured into cylindrical acrylic molds measure 1cm x 1cm. The molds were placed at 4°C for 3 hours to allow for full gelation to occur. In addition, new to this encapsulation procedure, the gels were then removed from their molds and individually placed in a solution of 0.1M $CaCl_2$ in a 10 mM citrate buffer at pH 3 for 5 minutes. Preliminary research showed that color leakage was lessened by this step, most likely to an association between the anthocyanin, pectins, and alginate. They were then placed in a clean 50mL cell culture flask that contained 25mL of 10 mM pH 3 buffer and then capped. Since each batch of gels was able to product multiple gels and since keeping them in a sealed container was necessary to prevent contamination, they were analyzed at various time points. (Table 4) The flasks containing the gels and citrate buffer (pH 3) were not opened until it was time for that particular gel to be measured. Control solutions were made by placing 103µL of the stock anthocyanin solution to match the anthocyanin concentrations in the gels in an identical culture flask with 25mL of pH 3 buffer.

	Gel Components							
	Water	Ethanol	Alginate	Slow Setting High-Methoxy Pectin	Rapid Setting High-Methoxy Pectin	Glucono δ-lactone	Anthocyanin	Other
Blueberry	85.68%	6.31%	1.07%	0.46%	0.46%	1.98%	0.01%	4.03%
Red Radish	85.74%	9.95%	1.09%	0.47%	0.47%	2.03%	0.01%	0.24%
Purple Corn	85.37%	10.18%	1.09%	0.47%	0.47%	2.03%	0.01%	0.39%

Table 3. Components and percent composition of the 3 different pectin-alginate particles that were created

2.2.2 Colorimetric Analysis

All color measurements were taken using a Hunter ColorQuest XE (Hunter Labs, Reston, VA). For all measurements, the CIE L*C*h color space, a 0.375" opening, D65 illuminant, and 10° observer angle were utilized. The color of the gel was measured using the reflectance specular included setting. After the gel was removed from the pH 3 buffer solution, the gel was placed in front of the 0.375" opening and a black cover was placed over the opening to prevent any light leakage. The color of the solutions during storage were monitored by placing the flask inside the machine and using the total transmission setting.

Particle	Solution Measurement Time points (days)	Gel Measurement Time points (days)	TMA Measurement Time points (days)
1	n/a	0	n/a
2	0, 0.083, 0.167, 0.25, 0.5, 1	1	1
3	2,3,4,5,6,7,8,9,10	10	10
4	15,20,25,30	30	30

Table 4. Study design demonstrating how long a gel was left unopened in its container and measurements that were taken on it.

2.2.3 Extraction of Anthocyanins from Gel

At predetermined time points of 1, 10 and 30 days, the flasks containing the gels were opened and gels were removed from the storage solution. The storage solution was condensed using a rotary evaporator. The gels were individually placed in 10mL of an acidified solution of 70% acetone (aq). The mixture was pureed for 10 sec using a Tissuemiser (Fisher Scientific, Hampton, NH) at 33,000 rpm. The puree was then centrifuged at 5,000g for 5 min. The resulting supernatant was collected and to the pellet a fresh 10mL of the acidified acetone was added, pureed and centrifuged. This was done for a total of 3 times, with the supernatants for each being combined. The combined supernatants were condensed using a rotary evaporator.

2.2.4 Spectrophotometric Analysis

Monomeric anthocyanin content was determined using the pH differential method (Giusti and Wrolstad 2001). A UV-Visible Spectrophotometer 2450 (Shimadzu, Columbia, MD) was used to collect spectral data at 520(508) and 700 nm with 1 cm path length disposable cells. Pigment content for purple corn and blueberry was calculated as cyanidin-3-glucoside equivalents, using a molecular weight of 449.3 and an extinction coefficient of 26,900 L cm^{-1} mg^{-1}. Red radish was calculated in pelargonidin-3-glucoside equivalents, using a molecular weight of 433.2 and an extinction coefficient of 31,600 L cm^{-1} mg^{-1}. The absorbance values are then used with the following equation to determine the total monomeric anthocyanin content TMA= A*MW*DF*1000/ε

2.2.5 HPLC Analysis

The anthocyanins from the pH 3 citrate buffer solution and those that were extracted from the gels were purified using a Sep-Pak® C18 Vac solid cartridge (20cc, 5g sorbent; Waters Corp., Milford, MA) was activated using methanol and then the extract was passed through. A red ring developed as the anthocyanins absorbed onto the column. A 0.01% HCl acidified water mixture was passed through the column eluting the sugars, acids and other soluble components. The anthocyanins were recovered via 0.01% HCl acidified methanol. The methanol was driven off via Buchi rotary evaporator (Buchi, Flawil, Switzerland) at 40C, to preserve molecular integrity, and the pigments were dissolved in acidified water.

Samples were analyzed using a high performance liquid chromatography (HPLC) (Shimadzu, Columbia, MD) system equipped with LC-20AD pumps and a SIL-20AC autosampler coupled to a LCMS-2010 Mass Spectrometer (Shimadzu, Columbia, MD) and a SPD-M20A Photodiode Array (Shimadzu, Columbia, MD) detectors. LCMS Solution Software (Version 3, Shimadzu, Columbia, MD) was used to analyze and graphically represent the analyzed data. A reverse phase Pursuit XRs C-18 column 3µm particle size measuring 4.6 ID x 150 mm (Agilent Technologies, Santa Clara, CA, USA) and a 4.6 x 22 mm Symmetry 2 micro guard column (Waters Corp. MA, USA) were used. Solvents and samples were filtered through 0.2µm Phenex RC membrane syringe filter (Phenomenex, Torrance, CA). Separation of all anthocyanins was achieved using a binary gradient: isocratic conditions at 9% for 7 min; linear gradient 9% to 11.5% B, 5

min; 11.5% to 11.5%, 2 min; 11.5% to 13%, 4 min; 13% to 21%, 7 min; 21% to 21%, 3 min; 21% to 40%, 2 min; followed by a return to initial conditions. Solvent A was 4.5% (v/v) formic acid in water(LC/MS grade) and B was 100% acetonitrile (LC/MS grade). The flow rate was set at 0.8 mL / min and an injection volume of 50µL was used. Spectral data was collected from 250-700 nm.

One fifth of the flow was diverted to the mass spectrometer. Mass spectrometry was conducted on a quadrupole ion-tunnel mass spectrometer equipped with electrospray ionization (ESI) interface (Shimadzu, Columbia, MD). Mass spectrometric analyses were performed under positive ion mode with the following settings: nebulizing gas flow, 1.5 L / min; interface bias, +4.50 kV; block temperature, 200 ºC; focus lens, -2.5 V; entrance lens, -50 V; pre-rod bias, -3.6 V; main-rod bias, -3.5 V; detector voltage, 1.5 kV; scan speed, 2000 amu / sec. A full scan (total ion count, TIC) was performed with a mass range from 200-1500 m / z and selective ion monitoring (SIM) was used to search for the molecular ions of the 6 common anthocyanidins (Table 3) throughout the analysis.

2.2.6 Statistical Analysis

R statistical software was used to perform a generalized additive model by non-parametric means. This analysis was performed on the transmittance of the solutions when comparing how they change during the first 5 days. Linear regression modeling was used when comparing each experimental solution to its control.

Minitab16 was used to perform analysis of variance (ANOVA) along with a Tukey's comparison of mean analysis at a 0.05 confidence interval. ANOVA was performed on the gel reflectance color measurements and the total monomeric anthocyanin content.

3 Results and Discussion

3.1 Anthocyanin Profile of Sources Used

3.1.1 Blueberry

The anthocyanin profile of the blueberry juice concentrate contains an assortment of anthocyanins. The published literature on the exact composition of blueberry varies from researcher to researcher. 13 anthocyanins were tentatively identified in the samples used and are listed in Table 5. Their identification was determined using published literature, order of elution, spectrophotometric and mass spectroscopy data. The wavelength of maximum absorbance, relative retention time compared to the others, mass of the aglycone, and mass of the entire anthocyanin were combined to give the tentative identifications. The blueberry juice concentrate contained anthocyanins from 5 of the 6 common aglycones: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Those 5 aglycones have the same 3 sugar moieties, galactose, glucose, and arabinose, which can be monoglycosylated. This gives a total of 15 possible anthocyanins. The chromatogram shown in Fig. 11 partially demonstrates the difficulty in developing an HPLC method that is capable of separating all 15 anthocyanins (if they were all there). This was more even

more difficult for this project due to the need to separate all three sources with the same method.

Anthocyanin	%*	Lambda max (nm)	Total m/z	Aglycone m/z
Delphinidin-3-galactoside	10.0	524	465	303
Delphinidin-3-glucoside	3.4	525	465	303
Cyanidin-3-galactoside	4.2	517	449	287
Delphinidin-3-arabinoside	5.0	525	435	303
Cyanidin-3-glucoside	1.5	517	449	287
Petunidin-3-galactoside	8.3	525	479	317
Cyanidin-3-arabinoside	1.9	520	419	287
Petunidin-3-glucoside	3.8	525	479	317
Peonidin-3-galactoside	1.7	520	463	301
Petunidin-3-arabinoside	3.2	528	449	317
Malvidin-3-galactoside	35.0	527	493	331
Malvidin-3-glucoside	10.4	528	493	331
Malvidin-3-arabinoside	10.7	529	463	331

Table 5. Anthocyanins identified from the source blueberry. * based on % area under the curve on the 520nm chromatogram.

3.1.2 Purple Corn

The purple corn anthocyanins were tentatively identified using the same technique as with the blueberry. The purple corn profile has 6 primary anthocyanins (Table 6). The first three are cyanidin, pelargonidin, and peonidin with glucose at the 3 position. The other three are those first three with a malonic attached to the 6 position of the glucose.

3.1.3 Red Radish

The anthocyanin profile of the red radish was the most different from the other two sources. (Table 7) All of the red radish anthocyanins that were tentatively identified are

derivatives of pelargonidin that is diglucosylated at the 3 position and monoglucosylated at the 5 position.

Anthocyanins	%	Lambda max (nm)	Total m/z	Aglycone m/z
Cyanidin-3-glucoside	55.3	516	449	287
Pelargonidin-3-glucoside	3.3	503	433	271
Peonidin-3-glucoside	15.0	516	463	301
Cyanidin-3-(6''-malonylglucoside)	20.5	519	535	287
Pelargonidin-3-(6''-malonylglucoside)	1.1	507	519	271
Peonidin-3-(6''-malonylglucoside)	4.8	519	549	301

Table 6. Anthocyanins identified from the source purple corn

Anthocyanins	%	Lambda max (nm)	Total m/z	Aglycone m/z
Pg-3-caffeoyl-soph-5-glu	3.1	506	919	271
Pg-3-caffeoyl-soph-5-malonyl-glu	7.9	507	1005	271
Pg-3-coumaroyl-soph-5-glu	10.8	506	903	271
Pg-3-feruloyl-soph-5-glu	8.4	506	933	271
Pg-3-coumaroyl-soph-5-malonyl-glu	38.9	507	989	271
Pg-3-feruloyl-soph-5-malonyl-glu	30.9	507	1019	271

Table 7. Anthocyanins identified from the source red radish. Pg: pelargonidin, soph: sophoroside, glu: glucoside.

3.2 Solution Color Changes

3.2.1 Comparison of sources to each other

As the anthocyanin laden gels were stored in the citrate buffer (pH 3), some of the anthocyanins leached out of the gel particles into the solution. This marked change in the color of the solution was easily noticed by the eye within a few hours. Since each anthocyanin source

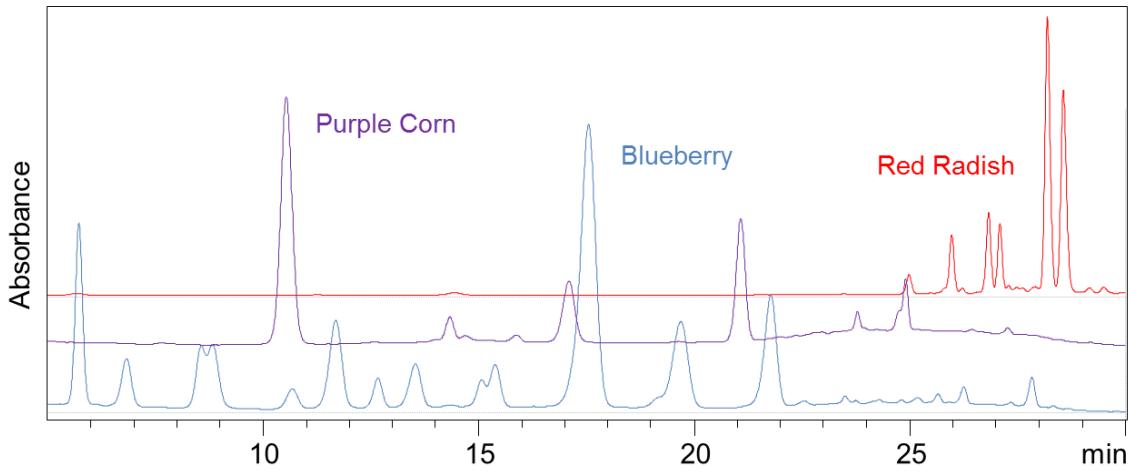

Figure 14. Stacked HPLC-PDA chromatograms demonstrating the prominent anthocyanins in the stock anthocyanin solutions measured at 520nm

would have different color parameters at the same concentrations, comparing the exact values (Appendix A) of the solutions would not yield relevant information. However, comparing the rate at which the parameters change relative to each other would prove more insightful.

The lightness of the red radish did not change dramatically during the study. Subtle variations were noticed during the first 12 hours, but the overall changes were minimal. This is represented in Figure 15 by the red radish line. The analysis of the rate at which the color changed resulted in a line that was almost flat. The blueberry lightness change occurred primarily during the first 5 days at a rate that was significantly faster than that of the red radish. The purple corn showed the most rapid changes during the first few days

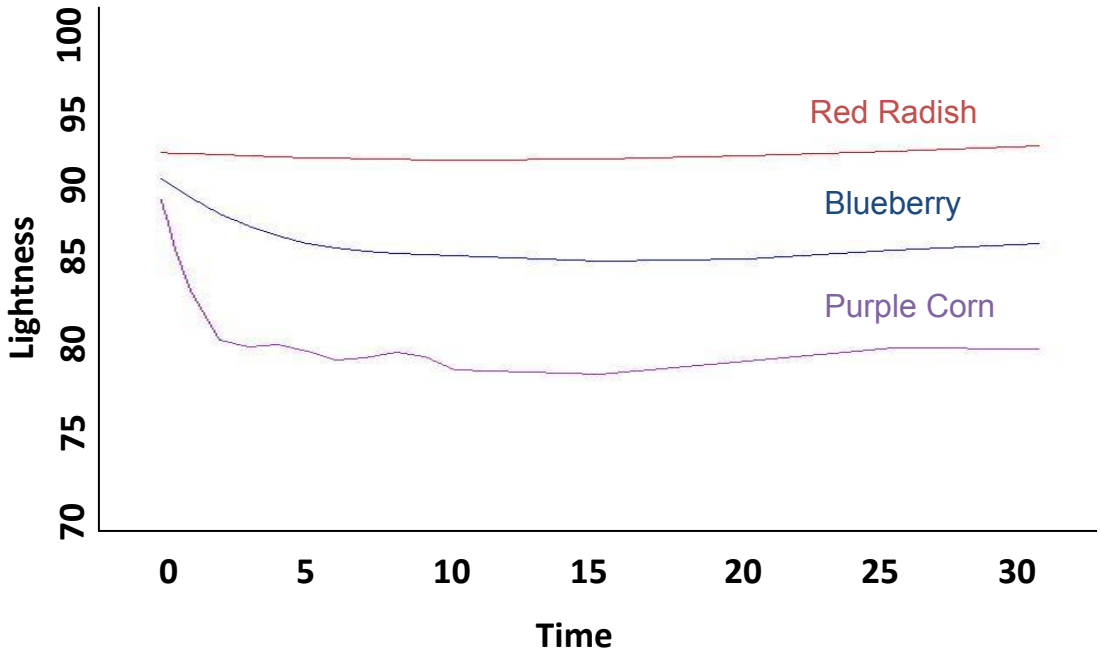

Figure 15. Regression lines from using non-parametric analysis to identify differences among lightness changes

as well. The solution became darker at a much significantly faster rate. A common occurrence for all of the samples was that after day 5, the lightness of the solution did not change significantly for the remainder of the study.

The intensity of the solution color, or chroma, had similar trends of the lightness except that these values increased over time instead of decreasing. In contrast to the lightness, the chroma of the solution containing the red radish gel did show a measurable increase during the first day. (Fig. 13) The increase in chroma was dramatic over the first 4 days and then reached a steady state for the remainder of the study. The purple corn also

demonstrated an initial period of rapid change followed by a steady state portion. The purple corn exhibited some fluctuations in the first 10 days as the analytical model attempted to compensate for minor changes. These changes were much less noticeable in the actual data but were emphasized in the model. The changes of the chroma in the blueberry containing solution followed a pattern almost identical to the red radish, except that it occurred slightly faster and had a greater maximum chroma. The rates at which the chromas changed were significantly different for all three of the anthocyanin sources.

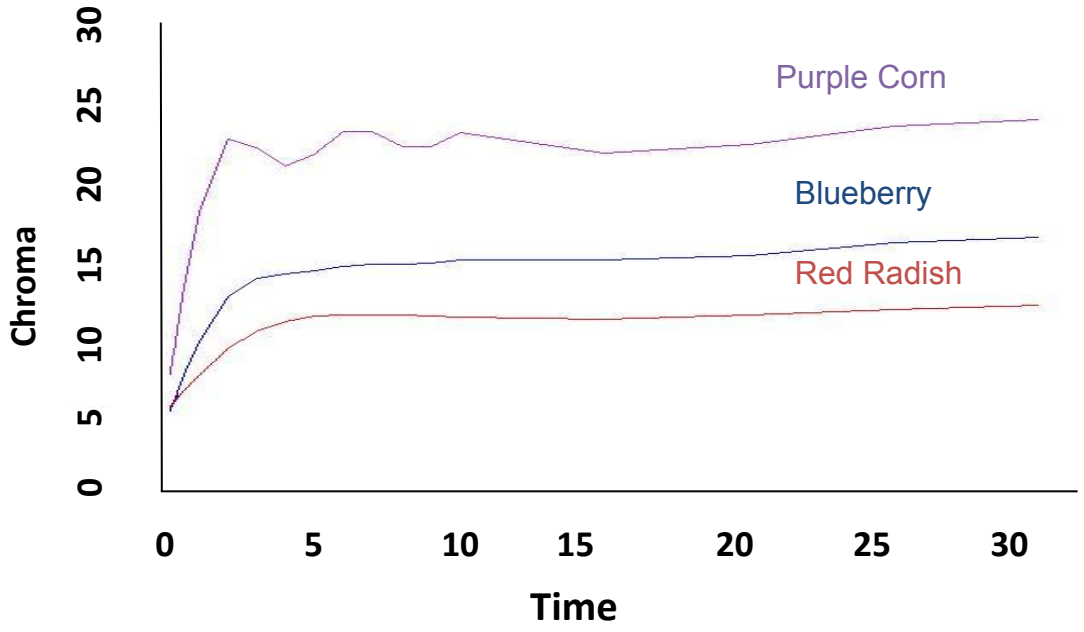

Figure 16. Regression lines from using non-parametric analysis to identify differences among chroma changes

The hue of the various solutions does not change nearly as much as the lightness or chroma did. The purple corn and blueberry containing solutions show almost no change during storage (Fig. 14). The red radish showed some considerable change in the first 4 hours, but almost no change after that. The rate of change between the 3 samples was significantly different at first, but was no longer so after 12 hours.

The color changes to the solutions that developed were caused by anthocyanins that leached out of the particles and into the citrate buffer (pH 3) solution. If no color change at all had been

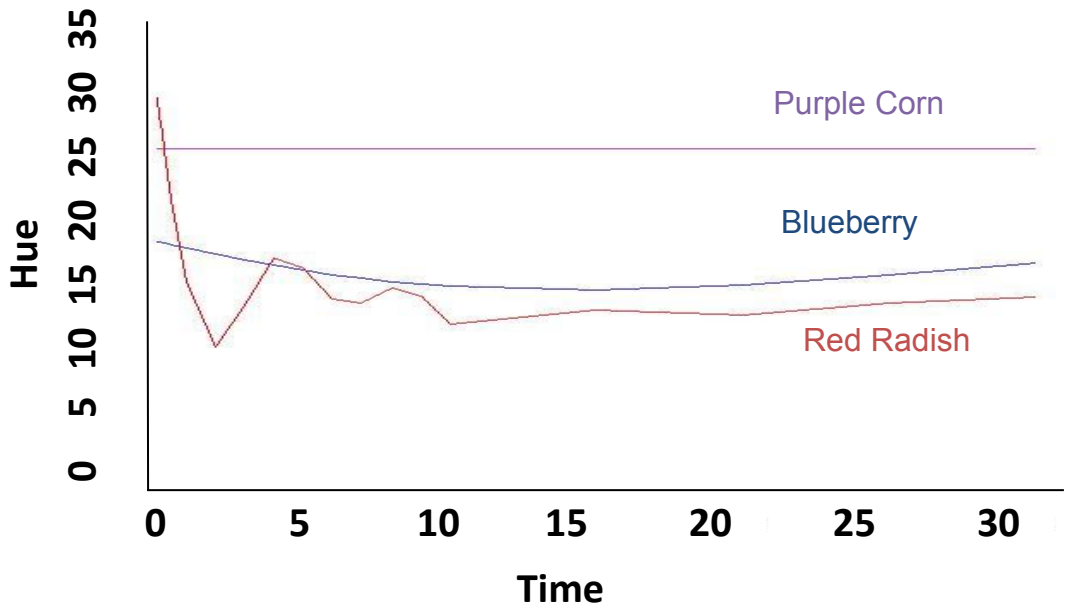

Figure 17. Regression lines from using non-parametric analysis to identify differences among hue changes.

noticed, that would have implied that the gels were able to perfectly encapsulate the pigments and prevent their leaching.

3.2.2 Comparison of Color Changes in Experimental and Control Solutions

The color changes that occurred during storage must be compared not only to each other, but also to the control solutions. The control solutions contained citrate buffer (pH 3) to which the anthocyanins were directly added at a level equivalent to what was in the gels. When these values are compared to each other, if the two values are significantly different, that would be considered a good thing. That means that some of the pigment was retained in the gels.

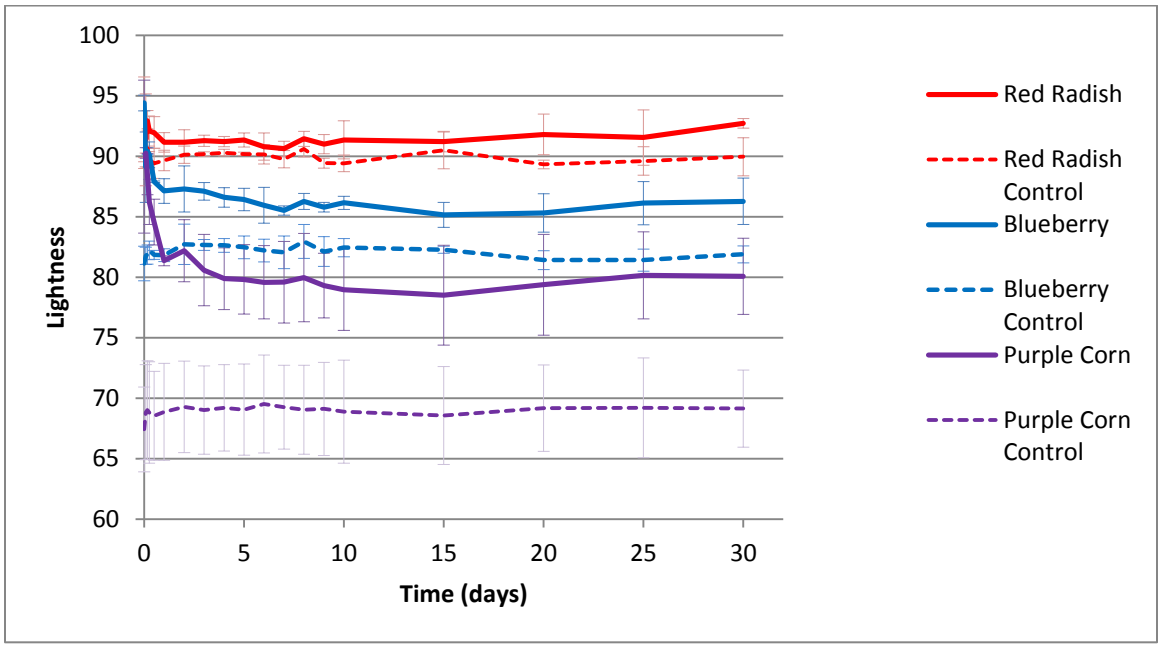

Figure 18. Lightness readings of the pH 3 buffer solutions that contained the gel particles and their respective control solutions.

The results of Figure 18 show the difference in lightness values. For all three anthocyanin sources, the control was darker than the experimental. The purple corn control solution had a lightness value that maintained fairly steady between 68 and 69 for the entire 30 days. The experimental purple corn lightness leveled off around 80 after a few days. This difference of more than 10 from the experimental versus the control was the largest of the three sources. The lightness of the blueberry control sample remained consistent around 82 throughout the study. The experimental blueberry lightness values stayed above 85 for the entire study. The red radish samples showed the least amount of difference between the control and experimental. The red radish control had a lightness of around 90 for the entire study. The experimental value was statistically significantly different, maintaining a lightness of around 92. For all of the control values, the lightness remains constant and level for the entire study. All of the experimental samples demonstrate a different trend. The lightness of the solution decreases over the first few days. The darkening of the solution ended prior to day 5 and the lightness value did not change significantly for the remainder, either up or down. Analysis of these changes over time was performed by using linear regression modeling. This analyzes and compares the entire line versus another instead of comparing individual points like is done with an ANOVA. Due to the initial changes that occurred in the experimental solutions, the data for t=0 through t=2 was omitted from these analyses. For all of these sources, the differences between the control and experimental were statistically different at a confidence interval of $p<0.01$. The red radish, while still being significant, did not produce a difference that was easily

noted by the human eye. This implies that the differences in these may not be relevant even though they are mathematically different.

The chroma, a measurement of how intense the color is, was also monitored and is shown in Figure 19. The chroma of the control solutions followed the same trend as the lightness wherein the value holds constant throughout the study. The major difference noted between the lightness and the chroma was that the chroma values of the control were greater than the experimental. Since a higher anthocyanin level will result in a darker solution that is more intensely colored, this difference makes total sense. The most

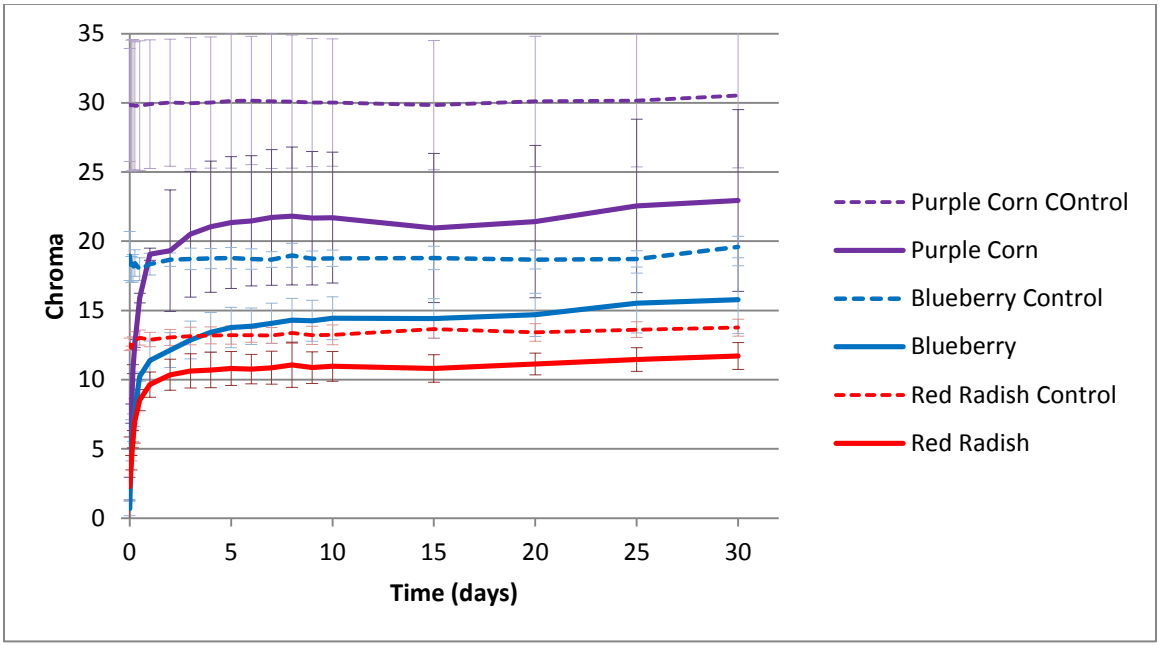

Figure 19. Chroma readings of the pH 3 buffer solutions that contained the gel particles and their respective control solutions

intense color was noted in the purple corn samples. The control had a consistent chroma of 30, a value that was more than 6 points greater than the experimental sample. The blueberry experimental samples were less vivid than its control, but the separation wasnot as great as with the purple corn. The control maintained a chroma of around 19 while the experimental stayed just below 15 for most of the study. The red radish had the least difference between the control and experimental. The chroma of the experimental sample stayed consistent between 11 and 12 while the control sample was between 13 and 14. The statistical differences, when applying the linear regression modeling, were all significant at $P < 0.01$.

Hue, the characteristic that best describes the shade of the color, was the most consistent parameter measured throughout the study (Figure 20). Using a confidence interval of 0.01, the purple corn was the only set of samples that were significantly different throughout the entire study. The blueberry and red radish samples were almost indistinguishable from their controls. These results were not necessarily positive or negative, but merely demonstrate that in some ways, the hue changed independently of the other color factors. The colors of the solution as compared to the control showed that some of the pigments were retained in the gel. This was made clearly evident by the separation of the experimental and control values for the lightness and chroma.

3.3 Particle Color Changes

As demonstrated by the differences for the color values of the solutions, some of the anthocyanins were retained in the gel particles. Directly measuring the color of the gels was therefore necessary to see if they were still able to provide color. Since any future use of these particles as a color agent would depend on the particles providing the color to the food, the direct color of the particles would be very important. At 1, 10, and 30 days, the gels were removed from their citrate buffer solutions for measuring. Once removed, gels were not placed back in the pH 3 buffer. After the color of the gels was measured, both the gels and their solutions were retained for further analysis.

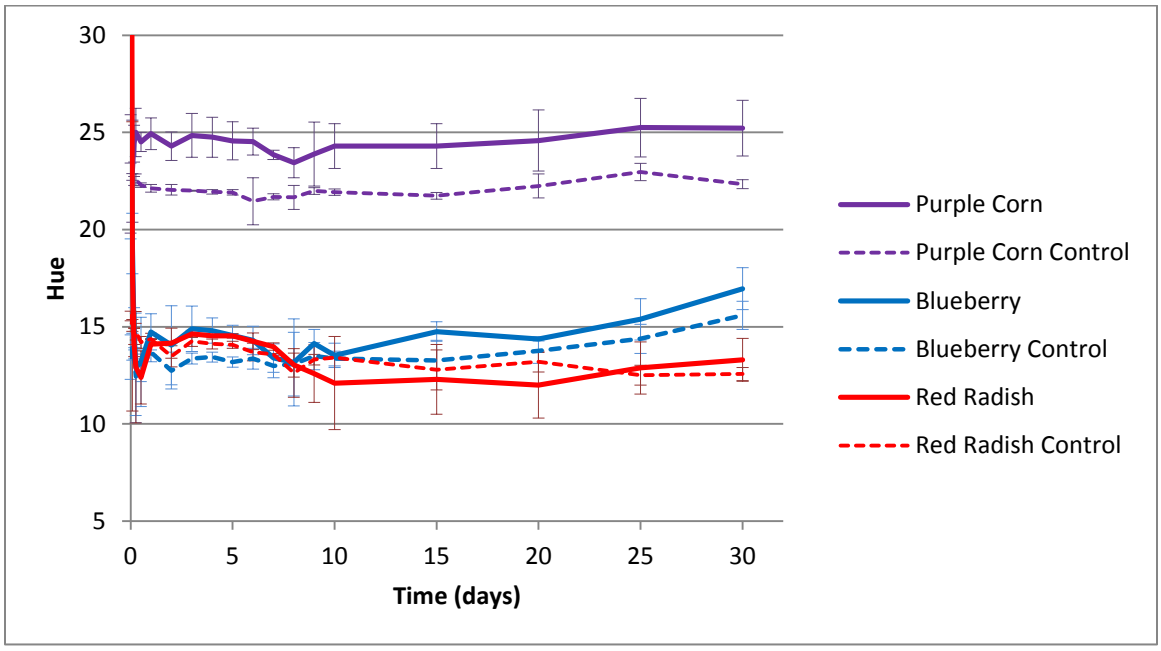

Figure 20. Hue readings of the pH 3 buffer solutions that contained the gel particles and their respective control solutions

The hue of the particles were noticeably different from one another. A gel with no color was considered the control. This allowed for a good baseline to compare them to. The purple corn changed the least, with a minor shift from 343 to 335 that occurred during the first day (Figure 21). The hue remained constant after the first day. The blueberry underwent a similar shift. The red radish had the most dramatic shift, starting at 7 and shifting to 310. All of these values were significantly different from one another using a confidence interval of 0.01. They were also significantly different from the empty control gel, which had a hue of around 250. The relatively minor changes observed in the gels were in contrast to the relatively larger changes in the solutions.

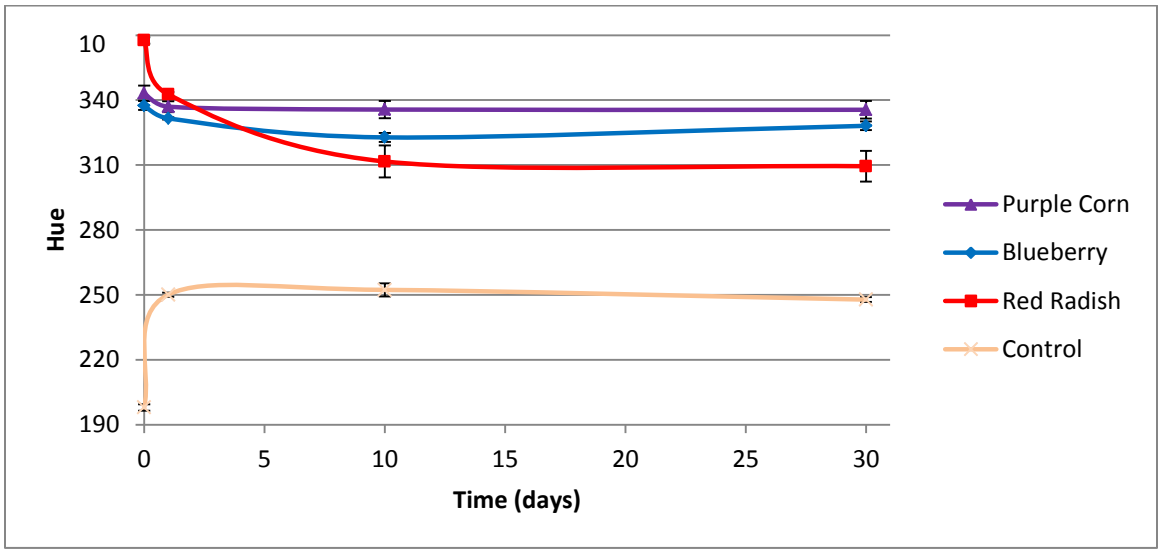

Figure 21. Changes in hue for the particles by measuring the particles directly

The color intensity of the gels was a very important characteristic to monitor (Figure 22). At day 1, all three of the samples were significantly different from the control. The blueberry and purple corn were the most intense and were not significantly different from one another, but were significantly different from the red radish. The results were noticeably different by day 10. The blueberry and purple corn were still indistinguishable from one another and had actually become slightly more vibrant. The red radish had lost almost all of its intensity. It was statistically no different from the control. These results were mimicked at day 30. The red radish was almost no different from the control while the blueberry and purple corn were much more intense. The blueberry and purple corn showed a non-significant increase in chroma over the 30 days, while the red radish chroma dropped by several points.

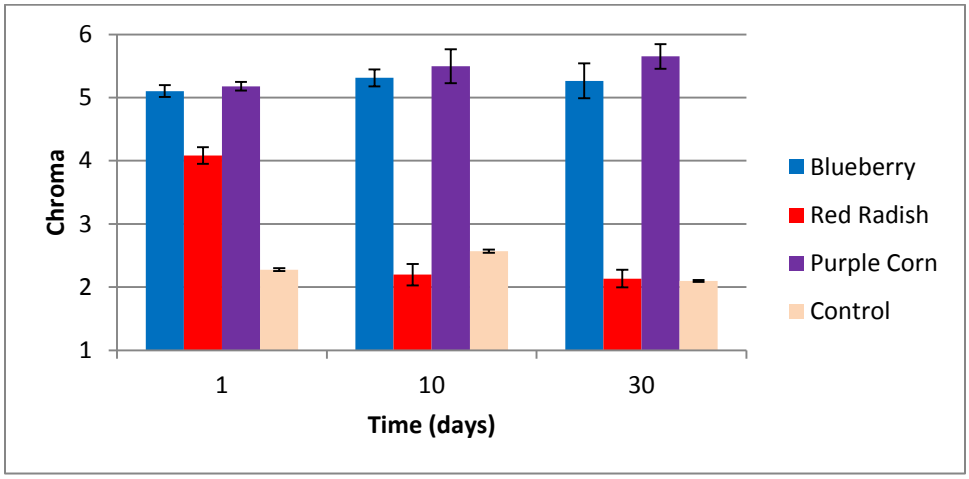

Figure 22.Chroma readings of the particles at 1, 10 and 30 days.

The lightness values of the gel particles were shown to vary over time. (Figure 23. Lightness readings of the particles at 1, 10 and 30 days. The lightness values at day 1 were similar to the chroma values at day 1, except that the values were inverted. The control particles were the lightest, with a value of around 28 that was maintained during the study. The lightness values at day 1 had blueberry and purple corn being not significantly different from one another while the red radish was significantly lighter. All of the anthocyanin loaded particles were significantly darker than the control. At day 10, the purple corn had not changed. The blueberry and red radish became significantly lighter. The red radish became so light that it was not significantly different than the control particle. These results held true at day 30 as well. The lightness readings for all 4 particles were not significantly different from day 10 to day 30.

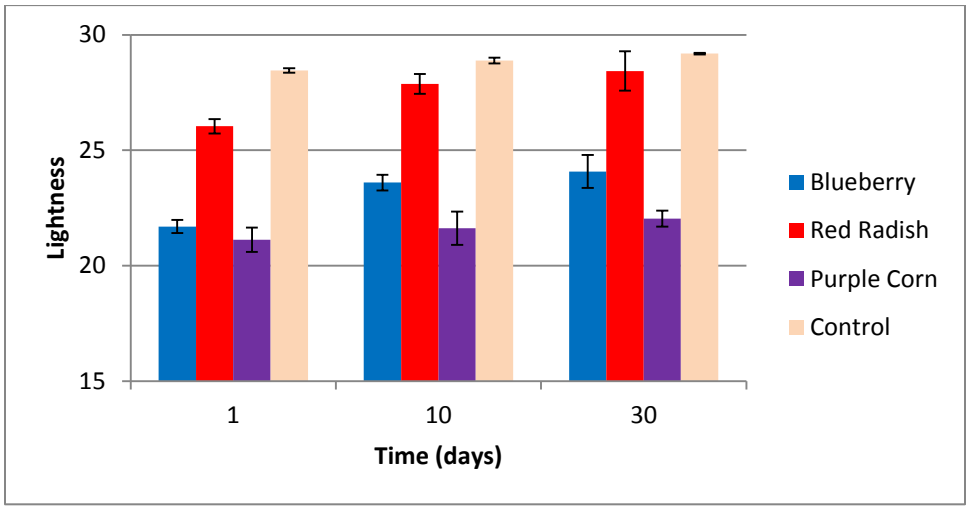

Figure 23. Lightness readings of the particles at 1, 10 and 30 days.

3.4 Anthocyanin Distribution Throughout Storage

3.4.1 Anthocyanin Extraction from Gel Material

After the color measurement were taken of the gels, it was necessary to remove the anthocyanins from the gel material. This needed to be done in a manner that was efficient, relatively quick, and would not destroy the compounds. The unique gelling characteristics of polymers used to make the particles proved a unique challenge. The anthocyanins needed to be removed from the wall material before they could be analyzed. The pH differential method, perhaps the most common technique for quantitatively analyzing anthocyanin content, requires the use of acidic conditions. If the particles had been simply placed in a neutral/alkaline solution until the polymers that comprise the particles disassociated, releasing the anthocyanins, simply reacidifying that solution would have created a semisolid liquid that could not be analyzed. Similarly, purifying the gels through a solid phase extraction method resulted in clogged cartridges that were prevented purification.

It was necessary to find an extraction solvent that the pigments would be soluble in while the alginate and pectins would not be soluble in it. Methanol and acetone were mixed with various amount of water and the particles were pureed with the mixtures. They were then centrifuged and the supernatant was collected. This was repeated several times to extract as much anthocyanin as possible. The collected supernatants were then able to be analyzed using the pH differential method. A mixture of 70% acetone was found to have

the best extraction levels because almost 100% of the anthocyanins that were put in the gels were able to be recovered. Methanol was only able to achieve 85% recovery.

3.4.2 Monomeric Anthocyanin Measurement

The gel particles were each loaded with ~103 µL of each respective anthocyanin rich extract. At t=0, 100% of the anthocyanins were located in the particle and 0% were found in the solution. After the first 24 hours, an unexpectedly high amount of the anthocyanin had leached into the solution.(Figure 24) For all three sources, more than 60% of the initial anthocyanin content within the particle was recovered from the citrate buffer (pH 3) solution. The percentage of the initial anthocyanin that were recovered in the solutions at Day 1 were not significantly different from one another. The amount recovered from the blueberry and purple corn gel at Day 1 was 24.4 & 26.0% respectively. The recovery from the red radish was significantly lower at 10.7%. At day 10, the levels that were recovered in the solutions ticked up slightly, to 72% for the blueberry and 68% for both the purple corn and red radish. These levels were not considered significantly higher than the levels from day 1. In a similar manner, the anthocyanins recovered from the particles went down, with less than 5% of the initial red radish anthocyanins recovered. The blueberry and purple corn fared slightly better, with 13% and 15% recovery respectively. The recoveries from the gels are significantly lower at day 10 as compared to day 1. The anthocyanin recovery at day 30 was very similar to that at day 10. The amount recovered in the solution decreased by a small, non-statistically significant amount.

An interesting note on the anthocyanin recovery can be noted. In all cases, the total amount of anthocyanin recovered by adding together the gel and solution amounts does not reach 100%. A logical conclusion could be made that this was due to pigment degradation during storage. This should have been unlikely to occur with the storage conditions at 4°C, at pH 3.0, and in a box that prevented any light from reaching them

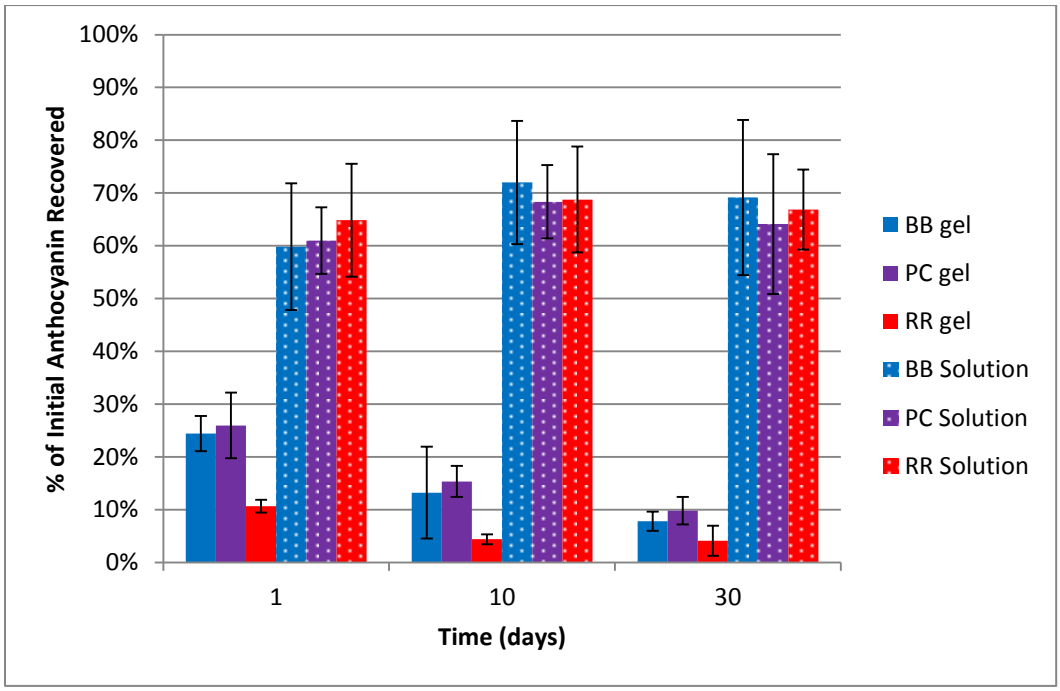

Figure 24. Total monomeric anthocyanin recovery

except for the brief times when they were taken out to be measured and then quickly put back in. To see if the storage conditions and experimental design had any effect on the anthocyanin breakdown, the control solutions that were used to compare the color caused by leaching also had their monomeric anthocyanin content measured. These solutions had greater than 90% of their anthocyanins recovered.

3.4.3 Anthocyanin profile change

As show in table 5, there were 13 different anthocyanins that were identified. The chromatogram in Figure 25 corresponds to the original blueberry extract that was used to create the particles. It was desirable to see if that relative composition of the anthocyanins would change during storage. The change could be as a result of breakdown, or possibly that some anthocyanins are more likely to remain in the gel and less likely to leach out. Most of the anthocyanins that make up blueberry were very minor relative to others. The 5 most prevalent pigments(Malvidin-3-galatoside, Malvidin-3-glucoside, Malvidin-3-arabinoside, Delphinidin-3-galactoside, and Petunidin-3-galactoside) were considered. (Figure 26) An ANOVA was performed for each individual anthocyanin to see if its

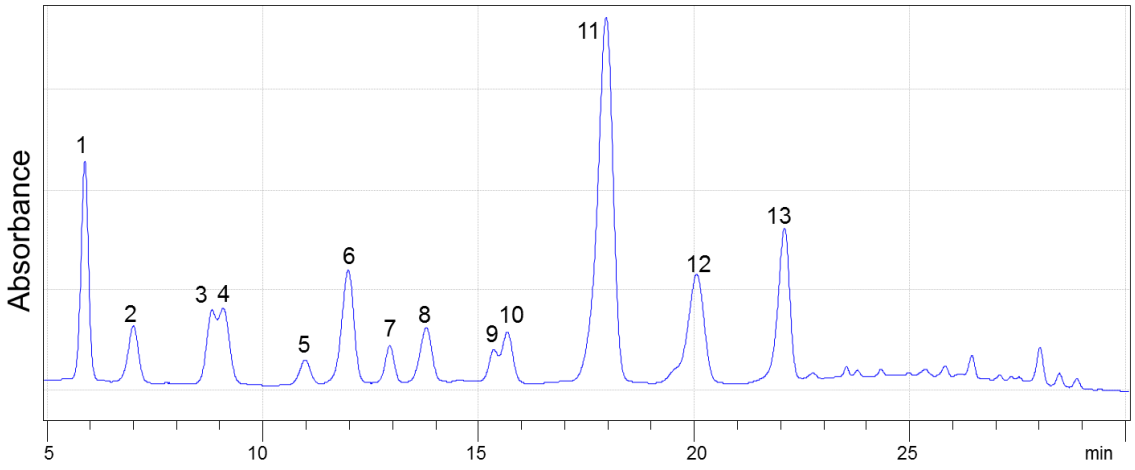

Figure 25. Chromatogram of the anthocyanin rich blueberry extract stock material monitored at 520nm. 1: Delphinidin-3-galactoside, 2: Delphinidin-3-glucoside, 3: Cyanidin-3-galactoside, 4:Delphinidin-3-arabinoside, 5:Cyanidin-3-glucoside, 6:Petunidin-3-galactoside, 7:Cyanidin-3-arabinoside, 8:Petunidin-3-glucoside, 9:Peonidin-3-galactoside, 10:Petunidin-3-arabinoside, 11:Malvidin-3-galactoside, 12:Malvidin-3-glucoside, 13:Malvidin-3-arabinoside

relative amount changed significantly throughout the study. The only one to show a significant different was for malvidin-3-arabinoside. The relative amount of it in the gel at 30 days was significantly greater than it was in the original material or the 30 day control solution. Other pigments showed marked differences, but due to their variability these differences were not statistically relevant.

The purple corn anthocyanins that were in Table 6 correspond to the chromatogram in Figure 27. Although there are 6 anthocyanins in purple corn, the 3 major ones (Cyanidin-3-glucosde, Peonidin-3-glucoside, Cyanidin-3-(6"-malonylglucoside)) were monitored more closely for changes. (Figure 28**)** None of the anthocyanins in purple corn had a significant change in percent composition. However, an interesting trend can be noted. The cyanidin-3-glucoside amount was greater for all of the day 30 treatments when compared to their day 1 counterparts. The opposite trend was noted for cyanidin-3-(6"-malonylglucoside). The other pigment levels remained relatively consistent for all measurements.

The red radish anthocyanins that were discussed from Table 7 are shown in the chromatogram in Figure 29. This was the anthocyanin profile form the stock red radish extract. Like the purple corn, red radish also has 6 anthocyanins that make it up, but the 6

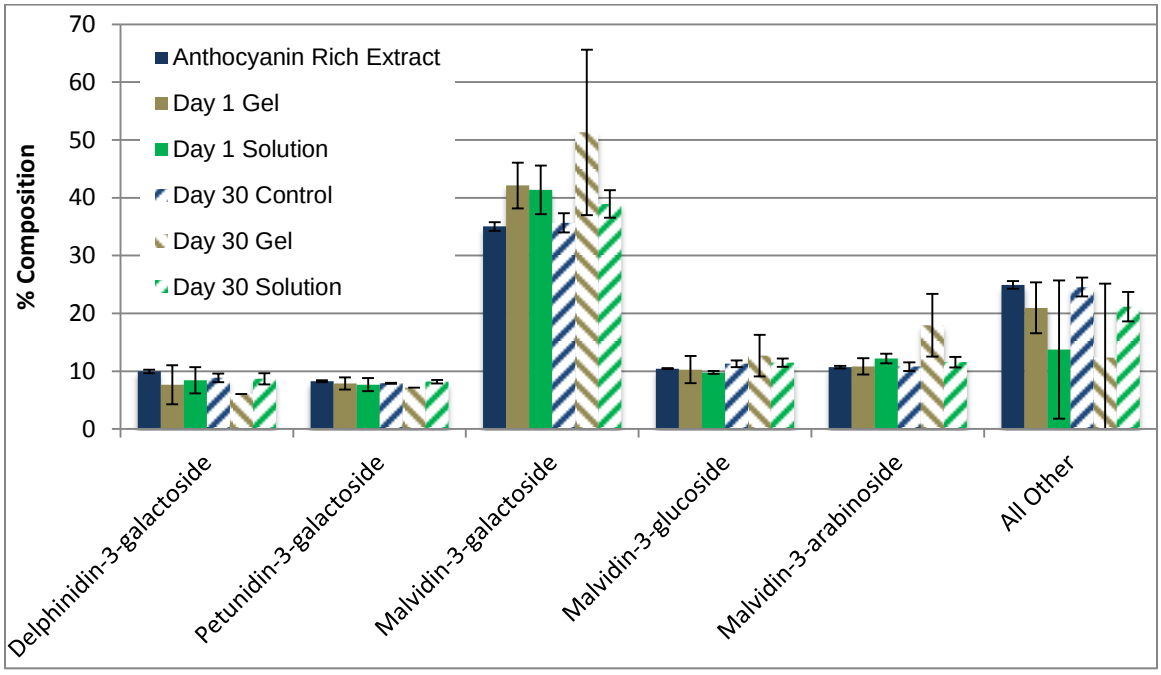

Figure 26. Relative amount of the anthocyanins in blueberry throughout the storage study

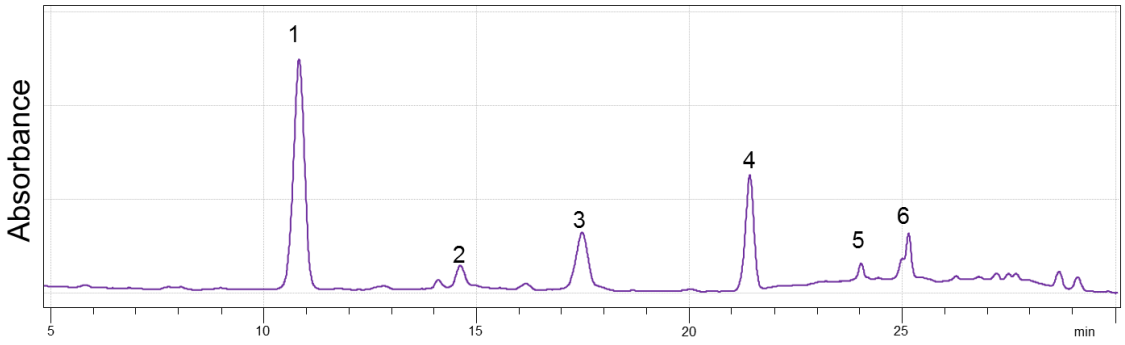

Figure 27. Chromatogram of the anthocyanin rich purple corn extract stock material monitored at 520nm. 1:Cyanidin-3-glucoside, 2:Pelargonidin-3-glucoside, 3:Peonidin-3-glucoside, 4:Cyanidin-3-(6"-malonylglucoside), 5:Pelargonidin-3-(6"-malonylglucoside), 6:Peonidin-3-(6"-malonylglucoside)

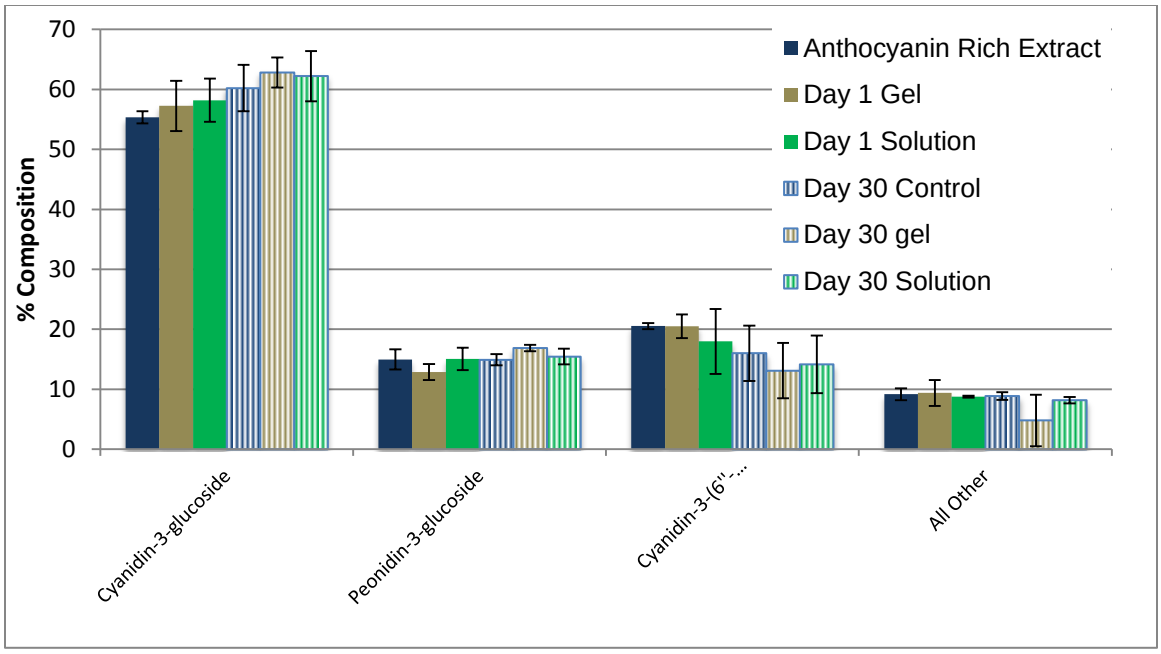

Figure 28. Relative amount of the anthocyanins in purple corn throughout the storage study

are very different. The red radish has the much larger pelargonidin derivatives with 3 sugar moieties and an aromatic acid attached. Since all 6 share a common backbone, the differences were as a result of the aromatic acid(caffeic, ferulic, coumaric) and whether or not a malonic acid was attached. The labels in Figure 30include only the acid information. Figure 30 also shows that only 4 of the 6 pigments were further investigated for differences. Much like the results for the purple corn, none of the individual anthocyanins in red radish showed a significant difference during the study. This was

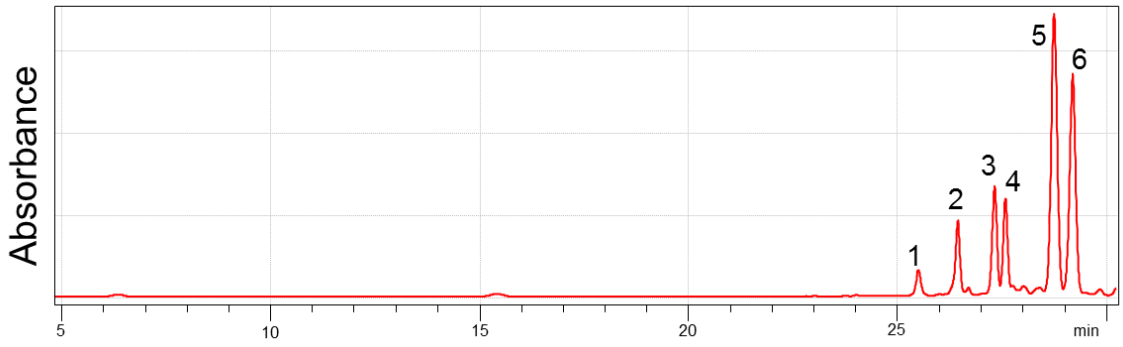

Figure 29. Chromatogram of the anthocyanin rich red radish extract stock material monitored at 520nm. 1: Pg-3-caffeoyl-soph-5-glu, 2:Pg-3-caffeoyl-soph-5-malonyl-glu, 3: Pg-3-coumaroyl-soph-5-glu, 4:Pg-3-feruloyl-soph-5-glu, 5: Pg-3-coumaroyl-soph-5-malonyl-glu, 6: Pg-3-feruloyl-soph-5-malonyl-glu

most likely due to the large variability. Even with the variability, a few interesting trends could be identified. The Pg-3-courmaroyl-soph-5-glu percent composition in both the day 1 and day 30 gels were greater than both the extract and the day 30 control. For the other pigments, the percent composition was greatest for the original extract than for any other sample.

In order to better understand the anthocyanin stability in the gel system, it was important to look at the specific anthocyanins that make up each extract. The blueberry extract has 5 of the 6 major aglycones, with pelargonidin being the only one that it is missing. The red radish extract only contains pelargonidin derivatives. It is possible that the structural differences between the pelargonidin with 1 hydroxyl group on its B-ring and the other aglycones with multiple hydroxyl or methoxyl groups?? Incomplete statement. There has been a very limited amount of research performed investigating the interactions between anthocyanins and pectins. (Buchweitz, Speth, Dietmar R Kammerer, et al. 2013;

Buchweitz, Speth, Dietmar R. Kammerer, et al. 2013) In these studies, some mixtures of pectin and anthocyanins were found to be more stable than just the anthocyanins themselves. Delphinidin and cyanidin were found to be more stabilized than pelargonidin. This stabilizing interaction could explain why the color of the blueberry and purple corn loaded gels was more intense and stayed more intense then the red radish gels, even though the amount of anthocyanin recovered from the gels and the citrate buffer (pH 3) was not significantly different.

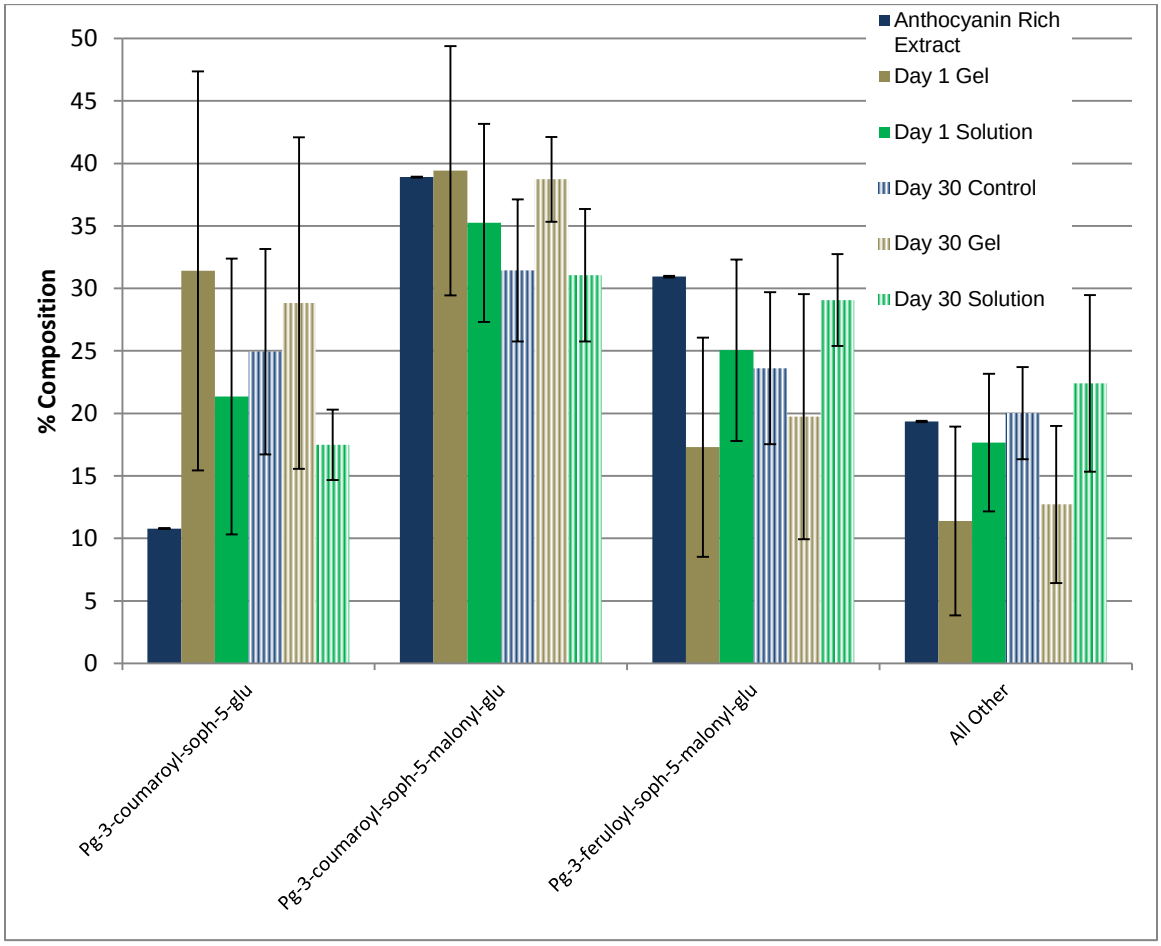

Figure 30. Relative amount of the anthocyanins in red radish throughout the storage study

The unique gelling properties of this system allowed the formation of a gel that was 1cm in diameter by 1cm tall, although any size and shape can be created with the proper mold. The color changes in the citrate buffer (pH 3) solution were as a direct result of the anthocyanins leaching out of the gel. All of the experimental solutions exhibited an increase in color, but the rates of increase were different for each sample. None of the experimental pH 3 buffer solutions that contained the gels reached the color characteristics of their controls. The purple corn samples were the most different from their control. The color loss of the gels during the 30 days of storage revealed some interesting insights. The hue of the various gels did not change dramatically while the lightness and chroma showed marked changes. When compared to a control gel with no anthocyanins loaded in it, the purple corn and blueberry loaded gels were significantly different for all time points. The red radish, however was almost indistinguishable from the control by day 30. Anthocyanin leakage into the pH 3 citrate buffer was clearly noted by the total monomeric anthocyanin measurements. The amount recovered from the solution at days 10 & 30 was greater than day 1, but this was not significantly greater and was this way for all anthocyanin sources. The total amount recovered from adding the gel and solution recoveries together never reached 100%. It is believed that this was due to an inability of recover all of the anthocyanins from the gels and not as a result of degradation. No preferential leaching in the solution or retention in the gel could be definitively identified. The lack of significant changes in the relative anthocyanin composition prevented this, although some specific trends could be noted in some of the anthocyanin sources.

Preliminary work in this lab investigated pelargonidin anthocyanins and the effect of acylation on stability in the gel. The results for pelargonidin with no acylating groups was poorer than for larger, acylated pelargonidin derivatives. These findings suggest that pelargonidin anthocyanins may not work well in this system, but further research needs to be performed to validate this hypothesis. It is also necessary to understand if the specific anthocyanin concentrations and relative ratio compared to the citrate buffer played a role in the stability. This can be investigated by loading more anthocyanins into the gels or place it in a different volume of citrate buffer.

4 Conclusion

Anthocyanins were successfully incorporated into a pectin-alginate gel. The combination of all the results demonstrates that simple anthocyanin structures present in the anthocyanin rich extracts from both purple corn and blueberry were suitable for this encapsulation system. The blueberry source was a juice concentrate that was more difficult to work with due to the high sugar content, but other blueberry sources might not be like this. The diacylated pelargonidin derivatives from red radish extract proved to be a very poor source for this system. Although the anthocyanin level recovered was not significantly different from the other sources, the color of the gel itself was lost much quicker than the other extracts used. This is in direct contradiction to the hypothesis and the reason for this is not understood at this time.

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Appendix A

	Blueberry 1			Blueberry 2			Blueberry 3		
time	L*	C*	h	L*	C*	h	L*	C*	h
0	95.14	0.34	47.77	94.07	1.29	17.52	93.98	0.44	40.82
0.083	85.92	5.76	20.14	89.76	7.05	20.22	91.62	4.07	17.49
0.167	90.73	7.34	13.83	90.15	6.85	14.88	90.34	5.42	16.00
0.25	90.72	8.89	9.75	88.91	8.13	13.74	90.77	6.50	15.19
0.5	87.94	10.46	11.57	87.84	9.98	14.82			
1	86.26	12.40	13.91	86.87	11.98	14.55	88.25	9.78	15.75
2	85.11	13.07	12.94	88.59	12.64	12.82	88.18	10.70	16.40
3	86.42	13.80	14.86	86.99	13.52	13.76	87.88	11.31	16.09
4	86.18	14.33	14.29	86.07	14.16	14.59	87.52	11.78	15.54
5	85.88	14.75	14.03	85.90	14.46	14.57	87.48	12.10	15.07
6	84.48	14.62	13.49	85.90	14.65	14.51	87.45	12.35	14.91
7	85.07	15.06	12.92	85.76	14.76	14.24	85.70	12.39	13.03
8	86.05	15.49	10.58	85.74	14.89	14.40	87.01	12.50	14.53
9	85.35	15.28	13.31	85.85	14.91	14.42	86.13	12.56	14.68
10	85.53	15.39	13.43	86.55	15.26	12.96	86.35	12.65	14.21
15	84.80	15.83	14.91	84.36	14.46	15.17	86.32	12.96	14.19
20	84.16	16.02	14.27	84.63	15.08	14.46	87.12	12.97	14.41
25	84.40	16.16	14.17	85.99	17.32	16.08	87.96	13.135	15.925
30	85.78	16.57	15.75	84.66	17.74	17.81	88.375	13.02	17.33

Table 8. Color measurements of blueberry used to create regression lines

	Purple Corn 1			Purple Corn 2			Purple Corn 3		
time (days)	L*	C*	h	L*	C*	h	L*	C*	h
0	93.41	1.96	30.58	82.67	3.03	25.54	93.82	1.42	29.68
0.083	89.80	6.99	20.08	90.33	7.32	25.18	90.67	8.32	23.75
0.167	86.73	11.10	25.41	90.12	10.46	24.35	88.705	10.735	23.51
0.25	84.43	12.89	25.07	86.08	12.31	26.20	88.135	12.655	23.725
0.5	82.78	16.22	24.90	84.30	15.52	24.69	86.545	15.935	23.95
1	80.90	19.49	24.68	81.49	19.10	24.28	81.68	18.595	25.845
2	81.12	22.53	23.48	80.31	21.12	24.49	85.11	14.305	24.91
3	78.58	23.40	24.50	79.19	22.86	23.93	83.965	15.27	26.115
4	78.09	24.11	24.30	78.75	23.46	24.02	82.79	15.595	25.93
5	77.76	24.43	24.25	78.58	23.76	23.79	83.11	15.87	25.66
6	77.39	24.53	24.09	78.30	23.83	24.17	83.03	16.05	25.33
7	77.51	24.74	24.12	77.77	24.38	23.72	83.47	16.065	23.7
8	77.54	24.87	24.21	78.19	24.53	23.46	84.15	16.07	22.665
9	77.58	24.98	24.50	77.96	23.88	22.02	82.37	16.135	25.145
10	77.74	25.07	23.80	76.38	23.74	23.49	82.78	16.31	25.615
15	75.98	24.38	24.02	76.29	23.76	23.31	83.28	14.76	25.555
20	76.07	24.50	23.69	78.00	24.70	23.66	84.055	15.08	26.4
25	77.77	25.97	23.79	78.41	26.37	25.15	84.285	15.32	26.8
30	77.85	26.91	24.68	78.66	26.58	24.13	83.665	15.375	26.835

Table 9. Color measurements of purple corn used to create regression lines

	Red Radish 1			Red Radish 2			Red Radish 3		
time	L*	C*	h	L*	C*	h	L*	C*	h
0	89.05	6.42	29.63	95.68	0.27	64.87	94.395	0.17	91.12
0.083	87.03	4.58	26.55	94.16	3.62	12.52	92.875	3.8	15.255
0.167	92.98	6.21	13.61	93.75	5.89	15.03	92.04	4.995	13.99
0.25	90.72	8.89	9.75	93.19	6.23	15.31	92.325	5.98	13.7
0.5	91.03	9.07	13.36	92.88	7.99	11.43			
1	90.23	10.65	13.50	91.71	9.46	14.22	91.495	8.845	14.605
2	90.01	11.54	13.31	91.48	10.23	14.80	91.99	9.305	14.36
3	90.77	11.93	14.65	91.40	10.50	14.82	91.66	9.465	14.45
4	90.73	12.05	14.56	91.34	10.57	14.58	91.545	9.5	14.455
5	90.72	12.11	14.57	91.41	10.62	14.63	91.88	9.675	14.405
6	89.49	11.88	13.76	91.41	10.65	14.58	91.48	9.775	14.42
7	90.09	12.02	13.82	91.30	10.94	14.18	90.465	9.63	13.945
8	92.06	12.83	12.39	90.82	10.82	13.07	91.43	9.58	13.635
9	90.82	12.10	12.54	90.29	10.71	14.10	91.845	9.81	11.14
10	90.75	12.17	13.39	90.15	10.64	13.59	93.13	10.09	9.34
15	90.63	11.82	14.30	90.82	10.81	11.77	92.19	9.82	10.83
20	89.86	11.86	13.93	92.75	11.27	10.83	92.775	10.28	11.22
25	88.91	11.95	12.37	92.74	11.96	14.41	92.985	10.48	11.875
30	92.41	12.35	12.94	92.59	12.20	14.55	93.15	10.585	12.425

Table 10. Color measurements of red radish used to create regression lines